Thermodynamics of Peptide Binding to the Transporter Associated with Antigen Processing (TAP)

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The ATP-binding cassette (ABC) transporter TAP plays an essential role in antigen processing and immune response to infected or malignant cells. TAP translocates proteasomal degradation products from the cytosol into the endoplasmic reticulum, where MHC class I molecules are loaded with these peptides. Kinetically stable peptide–MHC complexes are transported to the cell surface for inspection by cytotoxic T lymphocytes. The transport cycle of TAP is initiated by peptide binding, which is responsible for peptide selection and for stimulation of ATP-hydrolysis and subsequent translocation. Here we have analysed the driving forces for the formation of the peptide–TAP complex by kinetic and thermodynamic methods. First, the apparent peptide association and dissociation rates were determined at various temperatures. Strikingly, very high activation energies for apparent association ($E_a^{app} = 106 \text{ kJ mol}^{-1}$) and dissociation ($E_a^{diss} = 80 \text{ kJ mol}^{-1}$) of the peptide–TAP complex were found.

Next, the temperature-dependence of the peptide affinity constants was investigated by equilibrium-binding assays. Along with calculations of free enthalpy $\Delta G$, enthalpy $\Delta H$ and entropy $\Delta S$, a large positive change in heat capacity was resolved ($\Delta C = 23 \text{ kJ mol}^{-1} \text{ K}^{-1}$), indicating a fundamental structural reorganization of the TAP complex upon peptide binding. The inspection of the conformational entropy reveals that approximately one-fourth of all TAP residues is rearranged. These thermodynamic studies indicate that at physiological temperature, peptide binding is endothermic and driven by entropy.

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Introduction

Elimination of virus-infected or malignantly transformed cells by cytotoxic T-lymphocytes tightly depends on the transporter associated with antigen processing (TAP), which translocates peptides generated within the ubiquitin–proteasome pathway to MHC class I molecules in the endoplasmic reticulum (ER).\(^1,^2\) Peptide-loaded MHC complexes leave the ER via Golgi to the cell surface, where they are screened for their antigenic cargo by cytotoxic T-lymphocytes.

TAP belongs to the superfamily of ATP-binding cassette (ABC) transporters, which are characterized by the highly conserved Walker A and B motifs and the C-loop signature.\(^3\) ABC proteins are found in all three kingdoms of life and fulfill numerous (patho)physiological functions like drug export by the multidrug resistance protein (MDR1) or the cystic fibrosis transmembrane conductance regulator (CFTR) representing a chloride channel. All ABC transporters share a common modular architecture of at least four domains: two transmembrane domains (TMD) where each TMD contains five to ten transmembrane helices forming the translocation pore, and two conserved nucleotide-binding domains (NBD) that energize solute

Abbreviations used: ABC, ATP-binding cassette; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; CFTR, cystic fibrosis transmembrane conductance regulator; DTT, D,L-dithiothreitol; ER, endoplasmic reticulum; Fmoc, (9H-fluoren-9-ylmethoxy)carbonyl; GFP, green-fluorescent protein; HPLC, high performance liquid chromatography; PBS, phosphate-buffered saline; MDR, multidrug resistance; MHC, major histocompatibility complex; NBD, nucleotide-binding domain; Opp, oligopeptide permease; SDS, sodium dodecyl sulfate; TAP, transporter associated with antigen processing; TMD, transmembrane domain; single letter code, is used for peptide sequences.

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transport by binding and hydrolysing ATP. TAP is composed of two subunits TAP1 and TAP2, each consisting of one TMD and one NBD.

The transport mechanism of TAP can be dissected into two basic steps: (i) ATP-independent peptide binding and (ii) ATP-dependent peptide translocation.4,5 ATP binding does not require the presence of peptides.6,7 Recently we studied the kinetics of the peptide-binding step using peptides labelled with environment-sensitive fluorophores. These experiments demonstrated that peptide association induces a structural reorganization of TAP.8 Peptide binding is allosterically coupled to ATP-hydrolysis, indicating that TAP signals peptide binding to the NBDs.9 Recent structural work on P-glycoprotein,10 MsbA11 and BtuCD12 indicate that ABC transporters may undergo an extreme structural reorganization during solute transport. The structural changes in P-glycoprotein are mostly dependent either on ATP fixation or ATP-hydrolysis and are dependent on the translocation state of the transporter.10,13 In our present publication, we focused on the elucidation of the ATP-independent driving forces of peptide binding and subsequent structural reorganization of TAP by performing kinetic and thermodynamic analyses on the formation and dissociation of the peptide–TAP complex.

Results

Formation and dissociation of the peptide–TAP complex is marked by large energy barriers

The three-dimensional structure of a protein is determined by a network of hydrogen bonds, electrostatic and van-der-Waals forces. The structural reorganization of a protein implies rupture of these existing bonds before formation of a different set of bonds occurs, which stabilizes the new adopted structure. Since breaking of bonds requires energy, intensive conformational changes are expected to have large activation barriers. Therefore, we performed an Arrhenius analysis to determine the activation energy of peptide binding to TAP.

Association and dissociation kinetics of the peptide–TAP complex were analysed by a fluorescence quenching assay as established previously.8 In brief, TAP-containing microsomes were rapidly mixed with the fluorescein-labelled peptide, RRYC(-fluorescein)KSTEL, and the time dependent change of the fluorescence intensity was recorded. Peptide binding to the TAP complex induces fluorescence quenching by shifting proton donor groups in proximity to bound fluorescent peptide.8 In turn, addition of an excess of non-labelled peptide, RRYQKSTEL, leads to recovery of the initial fluorescence intensity reflecting dissociation kinetic of the peptide–TAP complex. The fluorescence changes strictly follow monoeXponential functions. The exponent yields the apparent association rate \( k_{\text{app}} \) and the dissociation rate \( k_{\text{diss}} \) for the assembly and dissociation of the peptide–TAP complex, respectively. At 283 K, the apparent association kinetics showed no linear but a hyperbolic dependence of the peptide concentration which is the normal behaviour of a two-step binding reaction with a fast peptide association followed by a slow conformational rearrangement that causes the fluorescence quenching. From these data, the overall affinity \( (K_D = 9.1 \text{ nM}) \) and second step \( (K_s = 0.4) \) as well as the dissociation \( (0.0029 \text{ s}^{-1}) \) rate constants of the slow structural rearrangements were determined.8 The dissociation rate constants \( (0.0021 \text{ s}^{-1}) \) measured by competition analysis resembles the slowest step of the whole reaction and is in good agreement with the dissociation rate constant of the structural rearrangement.

Using this methodology, the dissociation and apparent association rate constants of the peptide–TAP complex at 40 nM peptide were determined between 3 °C and 26 °C (276 K and 299 K). Experiments were performed in the absence of ATP to ensure that we analysed only conformational changes, which are induced by peptide binding and are not involved in subsequent translocation steps. For ATP depletion, microsomes were treated with apyrase, which was demonstrated to be sufficient to impede peptide transport by TAP.4,5,14,15 Above 26 °C and in the absence of ATP, the thermal deactivation of the TAP complex becomes the dominant process.16 As shown in Figure 1, apparent association and dissociation kinetics drastically increase by raising temperature. At temperatures examined, the kinetics can be described by monoexponential functions, as demonstrated by solid lines in Figure 1. For clarity, association and dissociation kinetics are shown only at 3, 14 and 26 °C. Table 1 summarizes all rate constants \( k_{\text{app}} \) and \( k_{\text{diss}} \). The data reveal a strong temperature-dependence of the formation and dissociation of the peptide–TAP complex. By raising the temperature from 276 K to 299 K the apparent association and dissociation rate constants increase by a factor of 44 and 12, respectively. The peptide-binding affinity and kinetics are not affected by ADP15 or non-hydrolysable ATP analogues, such as AMPPNP (Table 1). However, in presence of ATP, the dissociation and apparent association rate constants are decreased (Table 1).

In the next step, the apparent association and dissociation rates were examined by Arrhenius plots. Over the temperature range between 3 °C and 26 °C, Arrhenius plots for apparent association and dissociation are linear (Figure 2). Strikingly, very high activation energies for the formation of the peptide-TAP complex, \( E_a^\text{ass} = 106 \pm 6 \text{ kJ mol}^{-1} \), and for its dissociation, \( E_a^\text{diss} = 80 \pm 6 \text{ kJ mol}^{-1} \), were determined.
Peptide binding of TAP is accompanied by a large positive change in heat capacity

In addition to the kinetic studies, a thermodynamic analysis of equilibrium-binding constants can disclose distinct conformations of the TAP complex. Titration calorimetry is the method of choice to determine changes in standard free enthalpy $\Delta G$, enthalpy $\Delta H$, entropy $\Delta S$ and heat capacity $\Delta C$. However, this technique requires large amounts of protein, which must be stable for several hours. These requirements have not been achieved for TAP, even if microsomes from TAP-expressing insect cells are used. Therefore, we determined the thermodynamic parameters including $\Delta C$ via the temperature dependence of the dissociation constant $K_d$. In equation (1), $\Delta C^0_T$ at temperature $T_0$ and $\Delta C$ are used as fitting parameters. For convention, the standard free enthalpy change $\Delta G^0$ at pH 7.4 will be used throughout the text. The standard temperature $T_0$ is 25°C:

$$\Delta G^0_T = \Delta H^0_T - T \Delta S^0_T$$

$$= RT \ln K_d$$

Figure 1. Temperature-dependence of peptide association and dissociation. The association kinetics (upper panel) of the peptide–TAP complex were followed at 3, 14 and 26°C by mixing TAP-containing microsomes with 40 nM RRYC(-fluorescein)KSTEL. Fluorescence intensity ($\lambda_{\text{ex/em}} = 470/515$ nm) was recorded each three seconds. The dissociation kinetics (lower panel) were analysed after addition of a 400-fold molar excess of non-labelled peptide RRYQKSTEL. The experimental data (dots) are fitted by equation (11) or (12) (solid line).

Figure 2. Arrhenius analysis of the association and dissociation kinetics. The apparent association rate constants $k_{\text{app}}$ (filled squares) and dissociation rates $k_{\text{diss}}$ (open squares) were fitted by the Arrhenius equation (equation (13)). Activation energies $E_{\text{act}} = 106 \pm 6$ kJ mol$^{-1}$ ($R = 0.992$) and $E_{\text{diss}} = 80 \pm 6$ kJ mol$^{-1}$ ($R = 0.987$) were determined by linear fits. Error bars represent deviation of at least three independent experiments.

Table 1. Temperature-dependence of association and dissociation kinetics

<table>
<thead>
<tr>
<th>$T$ (K)</th>
<th>$k_{\text{app}}$ (10$^3$ s$^{-1}$)</th>
<th>$k_{\text{diss}}$ (10$^3$ s$^{-1}$)</th>
<th>Nucleotide</th>
</tr>
</thead>
<tbody>
<tr>
<td>276</td>
<td>2.7 ± 0.04</td>
<td>0.9 ± 0.02</td>
<td>No ATP</td>
</tr>
<tr>
<td>280</td>
<td>6.2 ± 0.1</td>
<td>1.2 ± 0.02</td>
<td>No ATP</td>
</tr>
<tr>
<td>284</td>
<td>9.2 ± 0.2</td>
<td>1.8 ± 0.03</td>
<td>No ATP</td>
</tr>
<tr>
<td>287</td>
<td>18.7 ± 0.6</td>
<td>2.6 ± 0.04</td>
<td>No ATP</td>
</tr>
<tr>
<td>291</td>
<td>26.1 ± 0.4</td>
<td>6.0 ± 0.3</td>
<td>No ATP</td>
</tr>
<tr>
<td>295</td>
<td>43.9 ± 2.3</td>
<td>7.8 ± 0.2</td>
<td>No ATP</td>
</tr>
<tr>
<td>299</td>
<td>119.0 ± 1.0</td>
<td>11.5 ± 0.4</td>
<td>No ATP</td>
</tr>
<tr>
<td>295</td>
<td>14.2 ± 0.2</td>
<td>4.3 ± 0.1</td>
<td>ATP</td>
</tr>
<tr>
<td>295</td>
<td>34.0 ± 0.6</td>
<td>8.6 ± 0.2</td>
<td>ADP</td>
</tr>
<tr>
<td>295</td>
<td>39.8 ± 0.8</td>
<td>7.1 ± 0.2</td>
<td>AMPPNP</td>
</tr>
</tbody>
</table>

The rate constants $k_{\text{app}}$ and $k_{\text{diss}}$ were determined at various temperatures by fitting the monoexponential equations (11) and (12) to fluorescence changes induced during association and dissociation of the peptide–TAP complex. Experimental details are given in the legend to Figure 1. Apyrase (no ATP), MgATP (3 mM), 3 mM MgADP (3 mM), or AMPPNP (3 mM) was added as indicated.
The dissociation constant $K_d$ can be derived either from kinetic analyses or from equilibrium-binding data, which are obtained from fluorescence changes $\Delta F_{eq}$ after reaching binding equilibrium at a given peptide concentration. Both methods yield identical $K_d$ values for peptide binding to TAP as reported previously. Since $K_d$ values derived from the equilibrium-binding assays ($\Delta F_{eq}$) are less error-prone, we focussed on this method. By varying the peptide concentration, $\Delta F_{eq}$ was recorded between 283 K and 299 K. The obtained $\Delta F_{eq}$ values were plotted against peptide concentration $[P]$ and fitted to equation (3). $\Delta F_{eqmax}$ represents maximal change of fluorescence at saturation of the binding sites:

$$\Delta F_{eq} = \frac{\Delta F_{eqmax}[P]}{K_d + [P]}$$  

All experiments were performed in the absence of ATP to ensure that exclusively the assembly of the peptide–TAP complex and not peptide transport was observed. As example, fluorescence changes $\Delta F$ and the peptide-dependent $\Delta F_{eq}$ values are shown at 292 K in Figure 3. At all temperatures examined, $\Delta F_{eq}$ values are in very good agreement with a typical Langmuir-type receptor–ligand (1:1) interaction. The equilibrium dissociation constants $K_d$ are summarized in Table 2. Interestingly, the $K_d$ values and subsequently the change in free energy $\Delta G^\circ_T$ (equation (2)) display a non-linear temperature-dependence. While the highest dissociation constant was observed at 16 °C (289 K; $K_{d289} = 58.0 \text{nM}$), nearly 3-fold reduced values for $K_d$ were found for both, the highest and the lowest temperature examined ($K_{d293} = 19.6 \text{nM}$, $K_{d299} = 20.3 \text{nM}$). The non-linear relation between temperature and $K_d$ is reflected in a minimal driving force for the formation of the peptide–TAP complex and subsequent structural rearrangement at 289 K. A large positive change in heat capacity $\Delta C^\circ$ of $22.6 \pm 4.8 \text{kJ mol}^{-1} \text{K}^{-1}$ was calculated by fitting the data to equation (1).

**Peptide binding to TAP is driven by entropy at physiological temperatures**

The driving force for every chemical process is given by a decrease in free energy ($\Delta G < 0$). Because $\Delta G$ is composed of $\Delta H$ and $\Delta S$ (equation (4)), the factor(s) driving the investigated process can be determined:

$$\Delta G = \Delta H - T \Delta S$$  

Both terms, $\Delta H^\circ_T$ and $\Delta S^\circ_T$ vary with temperature (equations (5) and (6)):

$$\Delta H^\circ_T = \Delta H^\circ_{T_0} + \Delta C^\circ(T - T_0)$$  

$$\Delta S^\circ_T = \Delta S^\circ_{T_0} + \Delta C^\circ \ln(T/T_0)$$

Besides the change in heat capacity $\Delta C^\circ$, equation (1) yielded the change in enthalpy $\Delta H^\circ_T$ and entropy $\Delta S^\circ_T$, at standard temperature $T_0$. At 298 K, values of $161 \pm 36 \text{kJ mol}^{-1}$ and $0.7 \pm 0.1 \text{kJ mol}^{-1} \text{K}^{-1}$, respectively, were obtained. Using these parameters in combination with equations (5) and (6), respectively, $\Delta H^\circ_T$ and $\Delta S^\circ_T$ were calculated (Table 2). Figure 4(b) shows $\Delta H^\circ_T$, $T\Delta S^\circ_T$, and $\Delta G^\circ_T$ plotted against the examined temperatures. Consequently, below 289 K, formation
of the peptide–TAP complex is an exothermic reaction with an unfavourable negative entropy term. Above 289 K, peptide binding is endothermic and driven by entropy (Figure 4(b)).

### TAP structure is dramatically reorganized upon peptide binding

Since change of entropy is crucial for TAP function, we analysed the entropy term in more detail. The overall change in entropy ΔS is the sum of opposing contributions and can be estimated by equation (7):

$$\Delta S = \Delta S_{HE} + \Delta S_{n} + \Delta S_{other}$$

(7)

ΔS<sub>HE</sub> reflects contributions caused by hydrophobic effects like moving apolar segments of the protein to water-accessible regions. ΔS<sub>n</sub> represents the loss of translational and rotational degrees of freedom upon binding and ΔS<sub>other</sub> contains all other effects including conformational transitions caused by peptide binding. In order to identify the contribution of individual terms, one can take advantage of temperature T, at which no change of total entropy $S^0$ is observed ($\Delta S^0 = 0$). This temperature was determined to be at 289 K as shown in Figure 4(b). $S^0_{HE}$ is expected to be temperature sensitive and can be estimated using equation (8). At 289 K a value $S^0_{HE} = -8.9 \text{ J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$ was calculated:

$$S^0_{HE} = 1.35 \Delta C^0 \ln(T/386)$$

(8)

The dependence of $S^0_{n}$ on temperature and mass are predicted to be logarithmic, meaning that $S^0_{n}$ should be relatively insensitive to size of the reactants and T. By determining $S^0_{n}$ for various ligand–protein interactions Spolar & Record obtained a value of $S^0_{n} = -0.2 \pm 0.04 \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$. Thus, knowing $S^0_{n}$ and $S^0_{HE}$, we calculate $S^0_{other}$ at $T = 289$ K. Equation (7) yields $S^0_{other} = 9.1 \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$. Since $S^0_{other}$, like $S^0_{n}$, is expected to be relatively temperature-independent, one can assume that these values are also roughly valid at physiological temperatures.

$S^0_{other}$ reflects the extent of substrate-induced structural reorganization. In case of rigid-body interactions lacking any conformational variation upon substrate binding usually negligible $S^0_{other}$ values are found. For example, $S^0_{other}$ for binding of subtilisin inhibitor to the subtilisin monomer can be calculated to be $-0.04 \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$. Note that $S^0_{other}$ for the subtilisin inhibitor–subtilisin interaction is negative because heat capacity decreases during this process (ΔC < 0). Thus, the extremely large value of $S^0_{other}$ is in agreement with a significant conformational change upon peptide binding. Moreover, because $S^0_{other}$ is positive, a drastic increase in disorder is expected after formation of the peptide–TAP complex.

### One-fourth of TAP structure is reorganized during peptide association

If $S^0_{other}$ is entirely based on changes in conformational entropy, the number of residues $R$ involved in the folding transition can be estimated by equation (9):

$$R = \frac{\Delta S^0_{other}}{23 \text{J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}}$$

(9)

The validity of equation (9) was confirmed by comparing the number of involved amino acid residues calculated by equation (9) and determined by nuclear magnetic resonance (NMR) or X-ray data. While only six to nine residues move upon bion binding to avidin, 36 shifted or tilted amino acid residues were calculated for the interaction of class II MHC molecule E<sub>k</sub> with the T-cell receptor 2B4. Fifty to 100 amino acid residues participate in the hirudin-induced folding of thrombin, whereas 102–120 residues change their position during the dimerization of the λ cro repressor. As calculated for TAP, approximately 396 residues are involved in the conformational change induced by peptide binding. Since TAP1 and TAP2 contain 748 and 686 amino acid residues, respectively, this means that more than one-fourth of the residues participates in the structural rearrangement.

### Discussion

The TAP complex translocates a wide spectrum of protein fragments into the ER, where these peptides are loaded onto MHC class I molecules. Peptide-loaded MHC complexes leave the ER to the cell surface, where they are inspected by cytotoxic T-lymphocytes. The transport mechanism of
TAP can be divided in at least two key steps; the ATP-independent peptide binding followed by ATP-dependent peptide translocation.\(^4,5\) The formation of peptide–TAP complex is crucial for TAP function in three aspects: first, peptide association represents the initial step of transport cycle;\(^4,5\) second, peptide binding determines the overall selectivity of the transport process;\(^25\) and third, peptide association induces a TAP structure which is capable of performing ATP-hydrolysis and peptide translocation.\(^8,9,26\) However, little is known about structural events during the transport cycle of TAP. Recent structural work revealed that ABC transporters may undergo profound conformational changes during the transport process.\(^10–13\) In the case of P-glycoprotein, either fixation or hydrolysis of ATP seems to induce the most drastic structural change.\(^10,13\) The drug efflux pump P-glycoprotein possesses a high and low substrate-binding site resembling the states before and after substrate transport.\(^27\) However, in the case of the peptide transporter TAP, the peptide-binding affinity is independent of the transport cycle. In the vanadate-trapped state, there is no change in affinity (M. Chen, R.A. & R.T., unpublished results). Moreover, ADP\(^17\) or non-hydrolysable ATP analogues such as AMPPNP have no influence on the apparent association rate constants and dissociation rate constants. Only in presence of ATP the dissociation and apparent association rate constants are decreased, which may reflect peptide binding and subsequent transport. We used kinetic and thermodynamic methods to investigate the driving forces for formation and dissociation of the peptide–TAP complex in the absence of nucleotides. We found three lines of evidence that TAP undergoes a large structural reorganization upon peptide binding.

The first indication is given by the high activation energy for the formation of the peptide–TAP complex. The structural reorganization faces a large energy barrier, since non-covalent bonds have to be broken before protein regions can move from one position to another. Arrhenius analysis revealed activation energies for the apparent association and dissociation process of 106 and 80 kJ mol\(^{-1}\), respectively. For comparison, the binding of GR-flu to serotonin 5HT\(_2\) receptor requires 50 kJ mol\(^{-1}\).\(^28\) In order to open the chloride channel CFTR, an activation energy of 104 kJ mol\(^{-1}\) is necessary while for closing the channel only 10 kJ mol\(^{-1}\) are required.\(^29\)

A second, more direct line of evidence for a large structural reorganization of TAP is given by the large change of heat capacity ($\Delta C^\circ = 22.6 \pm 4.8$ kJ mol\(^{-1}\) K\(^{-1}\)) during the formation of the peptide–TAP complex. A positive $\Delta C^\circ$ is diagnostic for an enlarged hydrophobic protein surface.\(^10,30,31\) Thus, after peptide binding, TAP exposes a higher number of hydrophobic residues to the solvent. The large value for $\Delta C^\circ = 23$ kJ mol\(^{-1}\) K\(^{-1}\) emphasizes the remarkable extent of polarity change of the TAP surface. While the change in heat capacity caused by glucose binding to yeast hexokinase was determined to be $\Delta C^\circ = -0.2 \pm 0.5$ kJ mol\(^{-1}\) K\(^{-1}\),\(^32\) binding of S-peptide to S-protein yields $\Delta C^\circ = -1.8 \pm 1.1$ kJ mol\(^{-1}\) K\(^{-1}\) and dimerization of $\lambda$ cro repressor results in $\Delta C^\circ = -6.4 \pm 0.7$ kJ mol\(^{-1}\) K\(^{-1}\).\(^33\) A similar absolute value for $\Delta C^\circ$ is reported for the conformational change of

![Figure 4. Thermodynamics of the peptide association process.](image)
Thermodynamics of peptide-TAP complex formation

pepsinogen $\Delta C^\circ = -25.5 \text{ kJ mol}^{-1} \text{ K}^{-1}$. Although most receptor–ligand interactions are characterized by a negative heat capacity change, few exceptions are known. For example, calcium binding to one of the two binding sites of troponin C$^{34}$ and the binding of the antibacterial magainin peptides to neutral membranes$^{35}$ show a positive change of heat capacity. Thus, similar to the latter proteins, TAP exposes an increased area of hydrophobic groups to the solvent after the conformational reorganization.

As a third line of evidence, we calculated $\Delta S_{\text{other}}^\circ = 9.1 \text{ kJ mol}^{-1} \text{ K}^{-1}$ as a thermodynamic estimate for the number of residues involved in the folding transition. Negligible values of $\Delta S_{\text{other}}^\circ$ are expected for rigid-body interactions, while interactions that are coupled to protein folding show large $\Delta S_{\text{other}}^\circ$ values. According to equation (9), approximately 396 residues are involved in the formation of the peptide–TAP complex. Hence, approximately one-fourth of TAP is restructured upon peptide binding. By H/D exchange measurements with P-glycoprotein similar drastic dynamic changes are found where one-fifth of all amino acid residues are affected by trapping the protein in the ADP/P state.$^{13}$ In contrast to TAP, the binding and/or hydrolysis of ATP and not substrate binding induces a structural rearrangement of P-glycoprotein.$^{10,13}$

Finally, one has to keep in mind that TAP is embedded in the membrane during all experiments. Therefore, we cannot exclude that the large $\Delta C^\circ$ and $\Delta S_{\text{other}}^\circ$ values are in part caused by altered interactions between TAP and the surrounding lipids. However, considering the dramatic changes of $\Delta C^\circ$ and $\Delta S_{\text{other}}^\circ$ it is evident that the effects are not caused by the nonameric peptide alone. Thus, the thermodynamic data demonstrate that TAP undergoes an extensive reshaping at this step of the transport cycle, which involves either large conformational changes or effects driven by an altered protein–lipid interface.

Further insights are attained from the analysis of $\Delta H^\circ$ and $\Delta S^\circ$. At 298 K, large positive values of $\Delta H^\circ$ and $\Delta S^\circ$ were determined for the formation of the peptide–TAP complex (161 kJ mol$^{-1}$ and 0.7 kJ mol$^{-1}$ K$^{-1}$, respectively). For comparison, at 298 K the opening process of the chloride channel formed by CFTR causes only an increase in enthalpy and entropy of 90.0 kJ mol$^{-1}$ and 0.3 kJ mol$^{-1}$ K$^{-1}$, respectively.$^{29}$ The binding of T-cell receptor M222 to its peptide–MHC ligand at 298 K yields $\Delta H^\circ$ and $\Delta S^\circ$ values of $-96.1 \text{ kJ mol}^{-1}$ and $-0.2 \text{ kJ mol}^{-1}$ K$^{-1}$.$^{36}$ Considering the very large positive values for $\Delta H^\circ$ and $\Delta S^\circ$ at 298 K and taking into account that $\Delta H^\circ$ is related to formation, breaking and distorting of bonds while $\Delta S^\circ$ corresponds to the change in order, the following details about the conformational changes of TAP can be discussed. First, the large positive $\Delta H^\circ$ value indicates the loss of numerous noncovalent bonds after formation of the peptide–TAP complex. Second, disorder is generated demonstrated by the positive $\Delta S^\circ$ value. The endothermic peptide association is driven by entropy at physiological temperatures. Possible sources of entropy may be the release of protein-bound water or adoption of a more flexible structure. The first hypothesis appears to be attractive because water molecules may be involved in the promiscuity of TAP. As known for the oligopeptide-binding protein OppA, buried water molecules can play a critical balancing structural role in mediating the binding of diverse side-chains by serving as adapters that occupy volume not filled by the ligand.$^{37}$ Due to its small size and large dipole moment, water is an ideal molecule to occlude the space between the surface of a substrate-binding site and various ligands of different size, shape, and polarity. Moreover, its capacity to act both as hydrogen bond donor and acceptor provides flexibility in possible hydrogen-bonding arrangements in the binding pocket. With regard to the large increase in entropy during the peptide–TAP association, it appears unlikely that the entropy is exclusively generated by a release of buried water from the peptide-binding pocket. For comparison, for the binding of trimeric peptides to OppA $\Delta S^\circ$ was determined between 0.17 kJ mol$^{-1}$ K$^{-1}$ and 0.21 kJ mol$^{-1}$ K$^{-1}$.

In this process, four to seven water molecules are involved.$^{37}$ Thus, either water molecules participate in the formation of the peptide–TAP complex to a greater extent or the latter hypothesis, the adaptation of a more flexible conformation, has to be taken into account as well.

In summary, peptide binding causes a drastic structural reorganization of the TAP complex. During this process, TAP obtains a more hydrophobic surface and probably a more flexible structure. The extreme extent of the structural rearrangement implies that this TAP isomerization plays a crucial role within the transport cycle. The conformational change might trigger ATP-hydrolysis and peptide translocation of TAP. We propose that the striking promiscuity (peptide sequence and length) of TAP is caused by water molecules which act as molecular glue between various residues of the ligand and the residues of the peptide-binding site.

Materials and Methods

Peptide synthesis

Peptides were synthesized by solid-phase technique using conventional Fmoc-chemistry and labelled with fluorescein via cysteine residues as described previously.$^8$ In brief, cysteine-containing peptides were incubated with 1.2-fold molar excess of 5-iodoacetamido fluorescein (Molecular Probes, Eugene, USA) in PBS, 20% (v/v) dimethylformamide, pH 6.0 for two hours at room temperature. After purification by reverse-phase HPLC, the identity of peptides was verified by mass spectrometry.
Microsome preparation and peptide-binding assays

TAP1 and TAP2 were co-expressed in SF9 cells using the baculovirus expression system. After cell disruption using a manual homogeniser, microsomes were isolated by differential and density gradient centrifugation. All buffers used for preparation were supplemented with 50 μg/ml AEBSE, 1 μg/ml aprotinin, 150 μg/ml benzamidine, 10 μg/ml leupeptin, and 5 μg/ml pepstatin. Microsomes were stored in PBS supplemented with 1 mM DTT at −80 °C. Protein concentration was determined by OD₂₈₀ and MicroBCA (Pierce, Rockford, USA). TAP concentration was determined by measuring the maximal amount of specific bound peptide (B_{max}) using the heterogeneous peptide-binding assay. An increasing amount of fluorescein-labelled peptide, RRYC(-fluorescein)KSTEL, were incubated with TAP-containing microsomes (35 μg total protein) in 150 μl assay buffer (PBS, 1 mM DTT, 5 mM MgCl₂, pH 7.4) for 15 minutes at 4 °C. Microsomes were washed in 500 μl ice-cold assay buffer (12,000 g, eight minutes, 4 °C). The microsomal pellet was solubilised in PBS, pH 7.4 containing 1% (w/v) SDS. The amount of bound peptides was quantified by fluorescence emission (λex/ em = 470/515 nm). The fluorescence signal was corrected for light-scattering. Unspecific binding was determined in the presence of a 400-fold molar excess of non-labelled peptide RRYQKSTEL. The number of bound peptides at saturation conditions B_{max} (representing the amount of TAP molecules) was calculated by plotting the quantity of specific bound peptide B against peptide concentration [P]. Data were fitted by equation (10). Kd reflects the dissociation constant:

\[ B = \frac{B_{max}[P]}{Kd + [P]} \]  

(Kinetic measurements)

Peptide association and dissociation kinetics of TAP were analysed using a fluorescence quenching assay as described previously. TAP-containing microsomes and fluorescein-labelled peptide RRYC(-fluorescein)KSTEL were mixed directly in the cuvette in 1 ml assay buffer (PBS, 1 mM DTT, 5 mM MgCl₂, pH 7.4). Mixing has a dead time of six seconds, which was proven to be not critical for the kinetic analysis. Absence of ATP was guaranteed by adding 0.03 U/μl ayprase. Changes in fluorescence intensity reflecting the association of the peptide–TAP complex were recorded in three-second intervals (λex/em = 470/515 nm) using a FLUROLOG-3 spectrometer (Instruments S.A., HORIBA Group, Paris, France) equipped with a microstirring device. Light-scattering was reduced by means of double monochromators and slits of 3 mm. In addition, inner filter effects were bypassed by triangular cuvettes, which reduce the pathway of light through turbid solution. The temperature of the sample was controlled by a circulating bath. After reaching equilibrium indicated by a constant emission signal, dissociation reaction was induced by adding a 400-fold molar excess of peptide RRYQKSTEL. The protein and TAP concentration was 0.2 mg/ml and 8 nM, respectively. Because of the low TAP concentration, pseudo-first order conditions were maintained. For clarity the acquired fluorescence raw data were expressed as changes of fluorescence ΔF. The apparent association rate k_{app} and the dissociation rate k_{diss} were determined by fitting the time-dependent fluorescence emission to monoeponential functions (equations (11) and (12)).

\[ \Delta F = \Delta F_{eq}(1 - e^{-kt}) \]  

(dissociation)

\[ F = \Delta F_{eq}e^{-kt} \]  

(ΔF_{eq} represents the fluorescence-emission difference after reaching binding equilibrium. For the Arrhenius analysis, ln k_{app} and ln k_{diss} were plotted against 1/T and fitted to equation (13):

\[ \ln k = \ln A - \frac{E_a}{RT} \]  

(R represents the gas constant and A the preexponential factor.

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Supplementary Material comprising one Figure is available on IDEAL.