Thermodynamic Analysis of the Interaction between the 0.5β Fv Fragment and the RP135 Peptide Antigen Derived from the V3 Loop of HIV-1 gp120*

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The Fv fragment of the 0.5β monoclonal antibody has recently been constructed, expressed, and purified. It binds with nanomolar affinity to the immunogenic RP135 peptide that is derived from the principal neutralizing determinant of HIV-1 in the third hypervariable region of gp120. Here, we analyzed the temperature-dependence of binding of the 0.5β Fv fragment to the RP135 peptide and a series of mutants thereof. Our results show that there is almost complete enthalpy-entropy compensation in the effects of mutations in the peptide on binding to the Fv, indicating that the mutations do not change the binding mechanism. There is good correlation, for residues within the antigenic epitope, between mutational effects on $\Delta C_p$ and calculated values of $\Delta C_p$ based on the extent of burial of polar and non-polar surface areas of amino acids. The value of $\Delta C_p$ for the binding of the 0.5β Fv fragment to the wild-type RP135 peptide is found to be $-5.0 \pm 0.9 \text{ kcal} \text{K}^{-1} \text{mol}^{-1}$ in the presence of 0.1% Tween-20 but only $-0.1 \pm 0.9 \text{ kcal} \text{K}^{-1} \text{mol}^{-1}$ in its absence. This result has important implications for the successful application of the structural parameterization approach to predicting changes in heat capacity that accompany binding reactions carried out in the presence of detergent or protein-stabilizing agents.

Advances in antibody technology have made possible direct cloning of antibody genes from hybridomas or lymphocytes into plasmid vectors, thereby facilitating protein engineering studies upon their expression in bacteria (1–3). Using protein engineering methods, one may tailor the specificity of antibody fragments, enhance their binding activity, and humanize them for use in therapy (4, 5). Recently (6), we constructed, expressed, and purified the Fv fragment of the 0.5β monoclonal antibody. This antibody, which was raised against the envelope glycoprotein gp120 from the IIIB isolate of human immunodeficiency virus type 1 (HIV-1) (7), binds to the principal neutralizing determinant of HIV-1. The 0.5β Fv fragment binds with nanomolar affinity (6) to the immunogenic 24-amino acid long RP135 peptide, NNTKRSIQRGPGRAFVTIGKRG (8), which is derived from the principal neutralizing determinant of HIV-1 found in the third hypervariable region of gp120. Recent nuclear magnetic resonance (NMR) investigations defined a 16-amino acid long antigenic epitope from Lys5 to Ile30 in the RP135 peptide (corresponding to residues 312–327 in gp120) and showed that it forms an 8-residue turn upon binding to the 0.5β monoclonal antibody (9, 10). The crystal and solution structures of the 0.5β monoclonal antibody (or fragments thereof) without and in complex with the peptide antigen have not yet been solved. The structures of antibody fragments of two other monoclonal antibodies, 50.1 and 59.1, in complex with V3 loop-derived peptides have, however, been determined (11, 12). In both cases, the antibody fragments bind 7-residue-long epitopes in a β-turn conformation, as frequently found in other antibody-peptide interactions (13).

Analysis of the energetics of peptide-antibody interactions is complicated by the fact that peptide antigens often undergo large conformational changes upon binding. To date, the energetics of interaction between antibodies and HIV-1-derived peptide antigens have been little studied. Previously (6), we characterized the salt dependence of the interaction between the 0.5β Fv fragment and the RP135 peptide and measured the contribution of arginine residues in the peptide to the interaction. To obtain further structural information on the interaction between the 0.5β Fv fragment and the RP135 peptide, we decided to determine the temperature dependence of the binding constant of this reaction for wild-type peptide and six mutants thereof. Isothermal calorimetry experiments did not yield reproducible results, probably owing to aggregation and/or nonspecific binding. We therefore carried out a van’t Hoff analysis in the presence of the non-ionic detergent Tween-20. Our results show that there is enthalpy-entropy compensation in the effects of the mutations in the peptide on the binding to the Fv fragment, indicating that the mutations do not change the binding mechanism. Using van’t Hoff plots, one can estimate the change in heat capacity, $\Delta C_p$, associated with the binding reaction. We find that the value of $\Delta C_p$ depends strongly on the concentration of detergent. This finding has important implications for attempts to correlate values of $\Delta C_p$ with the amount of surface area buried in protein-ligand complexes (14, 15). Although such a correlation has been applied successfully to predict the amount of surface area buried in the interface of some protein-protein complexes (16–18), it has failed in the case of others (19–24). We suggest that this failure is due, in part, to the frequent use of protein-stabilizing agents or detergents in binding experiments.

EXPERIMENTAL PROCEDURES

Materials—Expression and purification of the 0.5β Fv fragment was achieved as before (6). The antigenic peptide TRKSIQRGPGRAFVTIGK and variants thereof were synthesized, purified, and characterized as described (6). The concentrations of the peptides were estimated using the 2,4,6-trinitrobenzenesulfonic acid method for determination of amines (25). Isopropyl β-D-thiogalactopyranoside was

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† The abbreviations used are: HIV, human immunodeficiency virus; NMR, nuclear magnetic resonance.

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obtained from Cambidge Corp., Illinois. All other analytical grade reagents were purchased from Sigma.

**Fluorescence Enhancement Titrations**—Fluorescence measurements of the 0.5β Fv fragment in the presence of different peptide concentrations were made as described previously (6). All the reactions were carried out in 50 mM sodium phosphate buffer (pH 7.5) containing different fixed amounts of the non-ionic detergent Tween-20. Phosphate buffers were prepared separately for each temperature using a pH value of 6.86 at 25 °C and a temperature-dependence coefficient, $\alpha p K_a / \alpha T$, of $-0.0028$ (26). The titrations were carried out at least in duplicate over a temperature range from 10 to 35 °C ($\pm 0.1$ °C) in a thermostatted cuvette.

**Differential Scanning Calorimetry**—Differential scanning calorimetry was carried out using the MCS instrument (Microcal Inc., Amherst, MA) at a scan rate of $1$ K min$^{-1}$. The concentration of the Fv fragment was 26 μM in 50 mM phosphate buffer (pH 7.5) without or with 0.05% Tween-20.

**Data analysis**—Determination of dissociation constants ($K_D$) between the Fv fragment and the different peptides from the fluorescence enhancement titrations was achieved as described previously (6). van’t Hoff analysis was carried out by plotting $\ln K_D (= \ln(1/K_D))$ as a function of the inverse absolute temperature ($1/T$). The data were fitted using Kaleidagraph (Version 2.1, Synergy Software (PCS Inc.)) to the following equation,

$$\ln K_D = -\frac{\Delta H_p}{R} \left(\frac{1}{T} - \frac{1}{T_0}\right) + \Delta C_p \frac{T - T_0}{R} + \Delta C_s \ln \left(\frac{T}{T_0}\right) + \ln K_{D,0}$$

(Eq. 1)

where $K_D$ is the association constant, $T_0$ is a reference temperature in degrees Kelvin, $\Delta H_p$ and $\Delta C_p$ are the association constant and enthalpy change at the reference temperature, and $\Delta C_s$ is the heat capacity change of the binding reaction. Estimates ($\pm$ S.E.) of the parameters $\Delta H_p$, $\Delta C_p$, and $\ln K_{D,0}$ were obtained from the fits. The changes in free energy of binding, $\Delta G_0$, and entropy, $\Delta S_0$, at the reference temperature of 298 K were calculated using the following relations,

$$\Delta G_0 = \Delta H_0 - T \Delta S_0 = -RT \ln K_{D,0}$$

(Eq. 2)

where $R$ is the gas constant, and all other notations are as before. Equation 1 is derived by combining Equation 2 and the following relations.

$$\Delta C_p = \frac{\partial \Delta H_p}{\partial T} = T \frac{\partial \Delta S_0}{\partial T}$$

(Eq. 3)

**Surface Area Calculations**—Solvent-accessible areas were calculated using the method of Lee and Richards (27) in the Homology package within Insight II (Biosym/MSI).

**RESULTS**

**Temperature Dependence of Binding of the Wild-type RP135 Peptide to the 0.5β Fv Fragment**—A linear dependence of the fluorescence on the Fv concentration at different temperatures (in the absence of peptide) indicates that dissociation of the Fv fragment into light and heavy chains is negligible (Fig. 1). The thermodynamic parameters of the interaction between the 0.5β Fv fragment and the RP135 peptide at different temperatures between 10 and 35 °C were determined in the presence of different concentrations of Tween-20 by monitoring fluorescence enhancement upon binding. Plots of the natural logarithm of the association constant as a function of the inverse absolute temperature were found to have increasingly more convex shape in the presence of increasing concentrations of the non-ionic detergent (Fig. 2). Convex plots are characteristic of processes with a negative change in heat capacity. The data were fitted using Equation 1 and 298 K as a reference temperature. A linear relationship is observed between the value of $\Delta C_p$, the concentration of Tween-20 with a correlation coefficient of 0.89 (Fig. 3). The value of $\Delta C_p$ in the absence of detergent is estimated by linear extrapolation to be $-0.1 \pm 0.9$ kcal K$^{-1}$ mol$^{-1}$.

**Differential Scanning Calorimetry of the 0.5β Fv Fragment**—Differential scanning calorimetry of the 0.5β Fv fragment was carried out in the absence or in the presence of 0.05% Tween-20 (Fig. 4). Of importance is that the apparent heat capacity of the Fv fragment is always less negative when Tween-20 is present.

In the absence of Tween-20, two transitions are observed that may correspond to the dissociation of the Fv fragment and the denaturation of the two chains. In the presence of Tween-20, a third transition is observed that may be due to different melting temperatures of the light and heavy chains when the detergent is present.

**Effects of Mutations in the Peptide on Binding to the 0.5β Fv Fragment**—Six variants of the RP135 peptide with the single amino acid replacements R4A, K5A, R8A, R11A, F17A, and T19A were used in this study. These mutations span the entire sequence of the RP135 peptide except for the conserved GPGR loop that is thought to be crucial for stabilizing the conformation of the peptide (12, 28). van’t Hoff plots were constructed for the binding of these RP135 variants to the 0.5β Fv fragment in the presence of 0.05% Tween-20 (Fig. 2). The changes in heat capacity upon binding of the mutant peptides to the Fv were found to be negative and relatively large in all of the cases (Table I). The effects of the replacements R4A, K5A, R8A, R11A, F17A, and T19A on the free energy of binding ($\Delta G_0$) of the wild-type ($\Delta G_0$(wild-type)) and the mutants ($\Delta G_0$(mutant)) were found to be $-0.7 \pm 1.1$, $-0.2 \pm 1.4$, $-2.6 \pm 0.9$, $-2.3 \pm 1.1$, $-1.8 \pm 1.0$, and $-2.0 \pm 0.9$ kcal K$^{-1}$ mol$^{-1}$, respectively. The free energies, enthalpies, and entropies of binding of wild-type and mutant peptides at the reference temperature of 298 K were calculated using Equation 2 and are given in Table I. The effects of the replacements R4A, K5A, R8A, R11A, F17A, and T19A on the enthalpies of binding at 298 K ($\Delta H_0$(wild-type) − $\Delta H_0$(mutant)) are calculated to be $2.4 \pm 3.2$, $-2.3 \pm 5.2$, $-8.9 \pm 1.1$, $-2.4 \pm 3.5$, $-0.5 \pm 2.3$, and $-9.1 \pm 1.3$ kcal mol$^{-1}$, respectively. The effects of the replacements R4A, K5A, R8A, R11A, F17A, and T19A on the enthalpies of binding at 298 K ($\Delta S_0$(wild-type) − $\Delta S_0$(mutant)) are calculated to be $7 \pm 11$, $-8 \pm 16$, $-16 \pm 4$, $-3 \pm 12$, $5 \pm 9$, and $-27 \pm 4$ cal K$^{-1}$ mol$^{-1}$, respectively. The effects of the replacements R4A, K5A, R8A, R11A, F17A, and T19A on the enthalpies of binding at 298 K ($\Delta G_0$(wild-type) − $\Delta G_0$(mutant)) are calculated to be $0.16 \pm 0.11$, $0.09 \pm 0.18$, $-4.14 \pm 0.04$, $-1.45 \pm 0.12$, $-1.86 \pm 0.09$, and $-0.90 \pm 0.04$ kcal mol$^{-1}$, respectively.

**Enthalpy-Entropy Compensation**—The entropies of binding of wild-type and mutant peptides to the Fv fragment at 298 K and 0.05% Tween-20 were plotted as a function of the respec-
Interactive enthalpies of binding (Fig. 5). The data were fitted to the linear equation,

\[ D_S = a + bD_H \]  

(Eq. 4)

**Fig. 2.** van't Hoff plots for the binding of wild-type and mutant RP135 peptides to the 0.5β Fv fragment. Binding experiments were carried out at different temperatures in 50 mM sodium phosphate buffer (pH 7.5) containing different concentrations of Tween-20 in the case of wild-type peptide (panel A) and 0.05% Tween-20 in the case of the mutants (panels B-D). For additional details, see “Experimental Procedures.” The data were fitted to Equation 1.

**Fig. 3.** Plot of the change in heat capacity upon binding of wild-type RP135 peptide to the 0.5β Fv fragment as a function of the concentration of Tween-20. Binding experiments were carried out in the presence of different fixed concentrations of Tween-20 as described under “Experimental Procedures.” A linear relationship is observed with a correlation coefficient of 0.89.

**Fig. 4.** Differential scanning calorimetry curves showing the change with temperature of the apparent heat capacity of the 0.5β Fv fragment in 50 mM sodium phosphate buffer (pH 7.5) without (-) or with (- - -) 0.05% Tween-20. The DSC curves shown were base-line corrected by subtracting the curve of the change with the temperature of the apparent heat capacity of buffer alone or buffer with 0.05% Tween-20 as appropriate.
Experimental Procedures. The values of the enthalpy and entropy changes are

where \( i \) stands for the \( i \)th peptide. A linear relationship is observed with a slope, \( \beta \), of 0.0026 (± 0.0004) K\(^{-1}\) and a correlation coefficient of 0.94. This result indicates almost complete entropy-enthalpy compensation.

Surface Area Calculations—The change in solvent-accessible area upon binding of the RP135 peptide to the 0.5β Fv fragment is due to (i) area buried within the peptide and (ii) area buried in the interface between the peptide and Fv in their complex. The area buried within the peptide is the difference between the solvent-accessible area of the free peptide in its bound and unbound (extended) conformations. The conformation of a shorter version (Arg\(^{2}-\)Ile\(^{20}\)) of the peptide in its bound state was recently determined by NMR methods (10). The solvent-accessible area of the free peptide in its bound conformation was calculated using the NMR-derived coordinates to be 2064.8 Å\(^2\) (of which 1048.7 Å\(^2\) are nonpolar and 1016.1 Å\(^2\) are polar). The solvent-accessible area of the shorter version of the free peptide in an extended conformation was calculated to be 2444.7 Å\(^2\) (of which 1364.7 Å\(^2\) are nonpolar and 1080.0 Å\(^2\) are polar). The area buried within the peptide upon binding to the Fv fragment is, therefore, 379.9 Å\(^2\) (of which 316.0 Å\(^2\) are nonpolar and 63.9 Å\(^2\) are polar). In the absence of a known structure of the complex, the interface area between the peptide and the Fv in the complex was estimated from the literature on other peptide-antibody complexes (13) to be about 1000 Å\(^2\) (of which about 500 Å\(^2\) are estimated to be nonpolar and 500 Å\(^2\) are polar).

**DISCUSSION**

The interaction between the RP135 peptide and the 0.5β Fv fragment was studied here by measuring the effects of mutations in the peptide on the temperature-dependence of the binding reaction. In principle, the mutations in the peptide may alter the binding mechanism, thereby considerably complicating the analysis of their effects. It can be shown that enthalpy-entropy compensation is a good indication for a single interaction mechanism (29). The almost complete enthalpy-entropy compensation observed here (Fig. 5) indicates, therefore, a single interaction mechanism of the different mutant peptides with the Fv fragment.

Heat capacity changes associated with peptide binding are usually in the range of −0.1 to −0.5 kcal mol\(^{-1}\) K\(^{-1}\) although some exceptions have been reported. There are several reasons for changes in heat capacity due to ligand binding. A positive contribution to the change in heat capacity is due to the decrease in configurational freedom of one or both of the molecules that form the complex. It can be readily shown that the change in heat capacity is proportional to the change in the number of hydrogen bonds between the partners. There are some exceptions have been reported. There are several reasons for changes in heat capacity due to ligand binding. A negative and relatively large contribution to the change in heat capacity arises from binding-induced desolvation of nonpolar groups that are exposed to water in the unbound state. Finally, the heat capacity might change because of changes in ionization of charged side chains although this factor generally has a relatively small contribution.

We have attempted to rationalize the change in heat capacity associated with the binding of the RP135 peptide to the 0.5β Fv fragment by using the previously described structure-based parameterization approach (30). According to this approach, the effect of changes in hydration and noncovalent interactions on the change in the heat capacity, \( \Delta C_{p,hyd} \), upon folding or binding (14, 15, 17, 31) can be expressed as a linear combination of contributions associated with changes in polar (\( \Delta ASA_{p} \)) and nonpolar (\( \Delta ASA_{np} \)) solvent-accessible surface areas, as follows,

\[
\Delta C_{p,hyd} = a(T) \Delta ASA_{p} + b(T) \Delta ASA_{np}
\]

where the coefficients \( a(T) \) and \( b(T) \) are the respective contributions per Å\(^2\) of nonpolar and polar areas that become solvent-exposed, which are equal to 0.45 + 2.63 \times 10^{-4} (T - 25) - 4.2 \times 10^{-5} (T - 25)^{2} and −0.26 + 2.85 \times 10^{-4} (T - 25) + 4.31 \times 10^{-5} (T - 25)^{2} in cal K\(^{-1}\) mol\(^{-1}\) (32). The change in solvent-accessible area upon binding of the RP135 peptide to the 0.5β Fv fragment consists of changes due to (i) area buried within the peptide and (ii) area buried in the interface of the peptide-Fv complex. The area buried within the peptide upon binding is 379.9 Å\(^2\) (of which 316 Å\(^2\) are nonpolar and 63.9 Å\(^2\) are polar). The contribution of the area buried within the peptide to \( \Delta C_{p,hyd} \) is calculated using Equation 5 to be −0.125 kcal K\(^{-1}\) mol\(^{-1}\) at 25 °C. In the absence of a known three-dimensional structure of the Fv-peptide complex, we estimated the size of the interface area by assuming that, in the complex,
about half of the solvent-accessible area of the peptide becomes buried (13). The area buried in the interface of the peptide-Fv complex is accordingly estimated to be about 1000 A^2 (of which about 500 A^2 are estimated to be nonpolar and 500 A^2 are polar) per molecule, and its contribution to DeltaC_{p,hydr} is, therefore, about −0.2 kcal K^−1 mol^−1. The overall contribution to DeltaC_{p} due to the change in solvent-accessible area buried within the peptide and in the interface of the peptide-Fv complex is equal to about −0.3 kcal K^−1 mol^−1. This value is in agreement, within experimental error, with the value of DeltaC_{p} in the absence of detergent estimated by linear extrapolation to be −0.1 (± 0.9) kcal K^−1 mol^−1 (Fig. 3).

The magnitude of mutational effects on DG and DeltaC_{p} is consistent with NMR investigations (9, 10) that mapped the antigenic epitope from Lys^5 to Ile^20, i.e. replacement of Arg^4 and Lys^5 by alanine is found to have relatively little effect on both DG and DeltaC_{p}, whereas replacement by alanine of residues within the epitope has a large effect on both DG and DeltaC_{p} (Table I). There is a good correlation between the mutational effects on DeltaC_{p} and calculated values of DeltaC_{p,hydr} using Equation 5 and the polar and nonpolar surface areas of amino acids (27), for residues within the antigenic epitope (Fig. 6). Due to the uncertainty associated with linear extrapolation to zero concentration of detergent, the relatively large errors in the experimental values of DeltaC_{p} and the likely possibility that some side chains within the epitope are not fully buried, an agreement between the values of the experimentally measured DeltaC_{p} and calculated changes in DeltaC_{p,hydr} upon mutation is not to be expected.

Discrepancies between measured and calculated values of DeltaC_{p} have been observed in the case of other systems (19, 20, 33), suggesting that the structure-based parameterization approach may apply to folding but not to binding processes. Our results suggest that the lack of correlation is due, at least in some cases, to the presence of detergents or protein stabilizing agents. Polyethylene glycol, sucrose, and Tween-20, for example, are often used in biological assays to prevent denaturation, aggregation, or nonspecific binding. Many of these protein stabilizing agents are excluded from the surface of proteins which, therefore, become preferentially hydrated when these compounds are present at high concentrations (34). Such preferential hydration is expected to augment effects on heat capacity due to desolvation, as observed here in the case of the denaturation of the 0.5β Fv fragment in the presence of 0.05% Tween-20 (Fig. 4). Likewise, in the presence of Tween-20, both the peptide and the Fv fragment are preferentially hydrated, and complex formation between them, therefore, involves a larger desolvation step that should be reflected by a relatively more negative change in heat capacity, as indeed observed in this study (Fig. 3). This may explain why unusually large negative heat capacity changes have been observed in several other binding studies in which detergents or protein-stabilizing agents were employed (21–23).

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**Fig. 6. Correlation between experimental and calculated mutational effects on the change in heat capacity of binding.** Calculated values of DeltaC_{p,hydr} were obtained using Equation 5 and the polar and nonpolar surface areas of amino acids in Ref. 27. Single letter notation for amino acids is used.