Pergamon

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Bioorganic & Medicinal Chemistry 11 (2003) 941–949

BIOORGANIC & MEDICINAL CHEMISTRY

Role of Solution Conformation and Flexibility of Short Peptide Ligands that Bind to the p56^{lck} SH2 Domain

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Received 26 July 2002; accepted 18 October 2002

Abstract—A general approach in drug design is making ligands more rigid in order to avoid loss in conformational entropy (ΔS_{conf}) upon receptor binding. We hypothesized that in the high affinity binding of pYEEI peptide ligands to the p56^{lck} SH2 domain this loss in ΔS_{conf} might be diminished due to preorganization of the fourfold negatively charged pYEEI peptide in the bound, extended, conformation. A thermodynamic analysis was performed on the peptides Ac-pYEEI-NH_2, Ac-pYAAI-NH_2 and Ac-pYGGI-NH_2 using surface plasmon resonance (SPR) competition experiments to assay affinity constants at different temperatures. To study the effect of solution conformation and flexibility a computational conformation analysis was performed from which low energy conformations in solution were calculated, and S_{conf} estimated. It was found that the calculated low energy conformations for especially the pYE moiety in solution resemble that in the bound state. In the calculated minimum energy conformation in solution isoleucine is bent towards the pY aromatic ring, the occurrence of such conformation is experimentally confirmed by NMR. The estimated values for S_{conf} of the EE- and AA-peptide were similar, suggesting no predominant role of preorganization of the solution conformation due to electrostatic repulsion. Apparently the thermodynamics obey the same entropy-enthalpy compensation relationship, which also was found to hold for other peptides and peptidomimetics binding to p60^{src} family SH2 domains. The implications of the results for drug design are discussed.

Introduction

Change in conformation and flexibility may involve an important energetic contribution to ligand binding and is therefore relevant for ligand design.[1] Especially peptide ligands are highly flexible, which may cause poor selectivity and unfavorable entropy change upon binding. The peptide sequence pYEEI (pY being phosphotyrosine) is preferred for binding to certain Src homology 2 (SH2) domains. This sequence contains four negative charges in a short fragment, which may decrease the flexibility of this moiety and may give rise to preorganization for binding due to electrostatic repulsion.[2,3] There is a major interest in designing SH2 domain ligands for therapeutic purposes (reviewed by Sawyer et al.).[4] The interest in designing pYEEI-derived ligands for Src family SH2 domains prompted us to study the role of solution conformation and conformational flexibility in binding of pYEEI-peptides to the p56^{lck} SH2 domain.

SH2 domains are functional modules that are frequently involved in signal transduction, where they have a role in aggregating phosphotyrosine sequences that are part of intracellular proteins or membrane bound receptors participating in signal transduction cascades.[5,6] The SH2 domains of the Src family bind with high affinity to peptides containing the sequence pYEEI, as demonstrated by a degenerate phosphopeptide library.[7] The structural basis for binding of the pYEEI sequence to SH2 domains has been provided by the crystal structures from complexes with the p56^{lck} and the p60^{src} SH2 domains.[8,9] These crystal structures reveal a very characteristic binding mode that can be denoted as a two-pronged plug engaging a two-holed socket,[9] because the pY residue is buried in a deep positively charged pocket and the I residue in a hydrophobic pocket. The EE motif lies across the surface of the protein and was

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PII: S0968-0896(02)00536-9

initially expected to contribute little to binding. However, later studies showed that the EE motif is equally important as the I.[10] There is evidence that the pY + 1 E residue is involved in interaction with a basic residue on the surface of the SH2 domain and that the pY + 2 E residue and the peptide backbone are involved in a hydrogen-bonding network with water molecules.[11]

A remarkable feature is that the pYEEI sequence is bound in an extended conformation, with the negative charges of pY and both E residues positioned at relatively large intervening distances. We hypothesize that the electrostatic repulsion between the negative charges is minimal in the prebound conformation giving rise to an extended conformation in solution that may be partly rigidified and preorganized for binding to the SH2 domain. This feature may be denoted as electrostatically driven conformational control of the peptide in solution. Such conformational control and effects on the binding has been previously suggested by Burke et al.[2] and Charifson et al.[3]

We performed a study with peptide sequences pYAAI and pYGGI next to pYEEI, and focused on the contribution of this electrostatic conformational control to solution conformation as well as flexibility in relation to binding energetics. Our approach comprised a thermodynamic analysis of the interaction of the peptides with the $p56^{lck}$ SH2 domain as assayed with surface plasmon resonance (SPR) competition experiments. Furthermore, a computational conformation analysis was performed to estimate the conformation and the conformational entropy of the peptides in solution. The computations were performed using a recently developed method for extensive Monte-Carlo searching for energy minima in the vast conformational hyperspace of peptides.[12] From this search the solution conformation as well as the solution conformational entropy (S_{conf}) was derived. Moreover, NMR experiments were performed, attempting to get experimental information on the solution conformation of these small peptides.

Using this multidisciplinary approach, we found that the calculated low energy conformations showed a hydrophobic aromatic–isoleucine interaction that was experimentally confirmed by NMR, and that the conformation of the pYE moiety in solution agrees with that in the bound state. The estimated conformational entropy in solution for the EE- and AA-peptide appeared to be similar, which indicates that conformational preorganization does not play a predominant role in binding. The binding entropy was very well correlated with the binding enthalpy, demonstrating that the overall entropic contribution upon binding is controlled by entropy-enthalpy compensation.

Results

To study the thermodynamic characteristics of peptides binding to the $p56^{lck}$ SH2 domain a van't Hoff analysis was performed, which requires binding constants at various temperatures. Surface plasmon resonance (SPR) competition experiments were performed to obtain thermodynamic binding constants for the interaction in solution.[13,14] The SPR signal is very sensitive to small temperature changes as can be seen by the SPR signal of the sample cell and the reference cell at 40 °C (Fig. 1). The reference signal shows that it takes some time before temperature equilibrium is reached, however the net signal is not affected by these temperature effects and can be used for determination of binding constants. As an example, the results from a competition experiment at 40 °C are shown in Figure 2.

The thermodynamic binding constants for the interaction in solution were obtained by non-linear curve fitting of such data as described before.[13] The obtained data for the various peptides are represented as van't Hoff plots in Figure 3. Fits of the data to the integrated

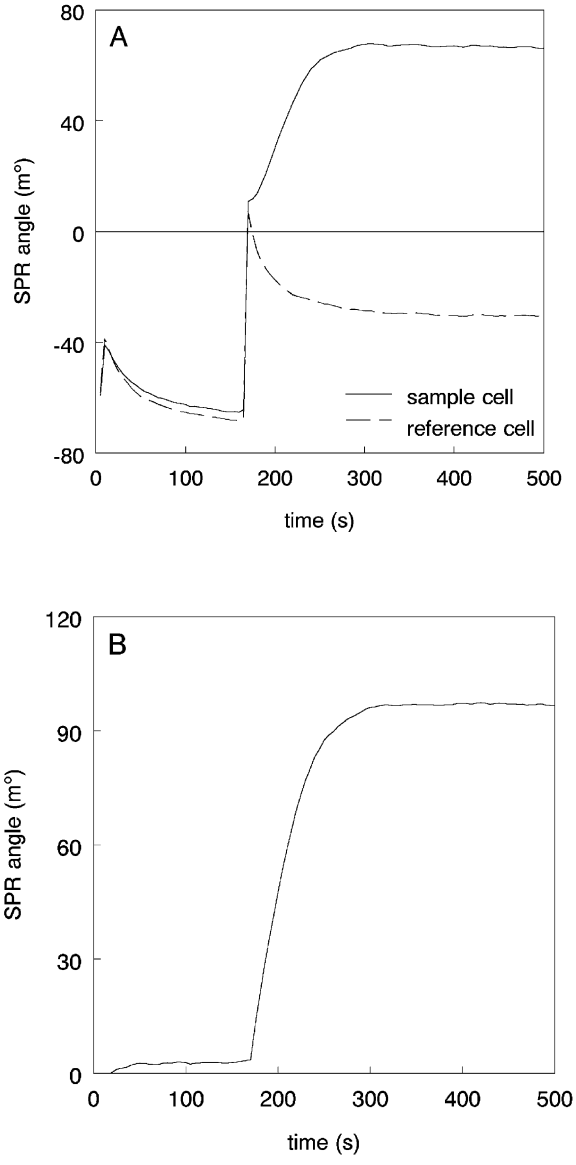

Figure 1. Temperature effect on SPR sensorgrams for binding of 50 nM $p56^{lck}$ SH2 domain to immobilized Ahx-pYEEI 11-mer peptide at 40 °C. Panel A: the signal of the sample cell (solid line) and of the reference cell (broken line). Panel B: the net signal (sample cell and reference cell subtracted).

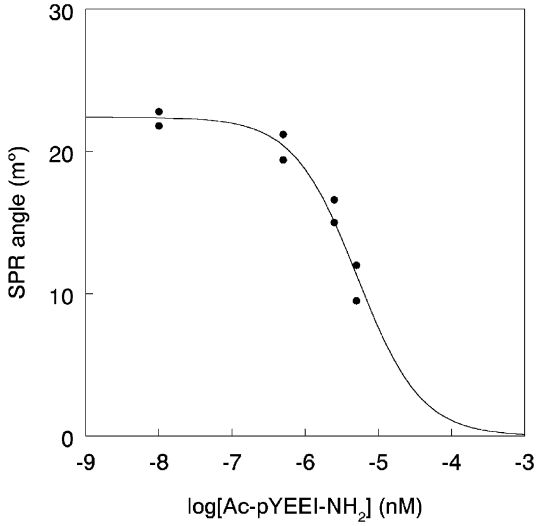

Figure 2. Surface plasmon resonance (SPR) competition experiment. The SPR signal at equilibrium for binding of 50 nM $p56^{lck}$ SH2 domain to an immobilized Ahx-pYEEI 11-mer peptide was determined in the presence of various concentrations of Ac-pYEEI-NH_2 peptide at 40 °C.

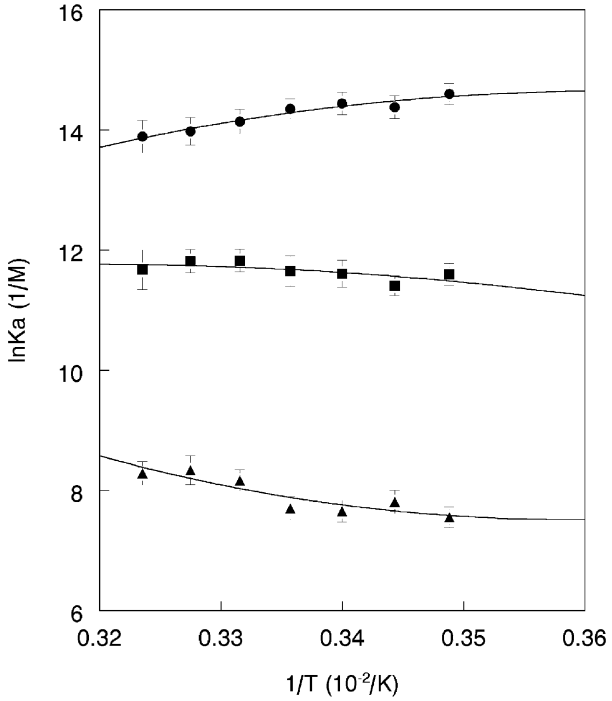

Figure 3. Van't Hoff plots for binding of pYEEI-derived peptides to $p56^{lck}$ SH2 domain. The affinity in solution for Ac-pYEEI-NH_2 (●), Ac-pYAAI-NH_2 (■) and Ac-pYGGI-NH_2 (▲) was obtained from SPR competition experiments. The error bars indicate the standard error in the affinity data.

van't Hoff equation (Eq. 1) yields the thermodynamic parameters as presented in Table 1. As thermodynamic analysis using SPR is not a common practice, we first evaluated our results by comparing them to published results. The SPR derived thermodynamic parameters for Ac-pYEEI-NH_2 compare very well with data for this peptide binding to the $p60^{src}$ SH2 domain as obtained from ITC experiments (ΔH° and $T\Delta S^\circ$ −5.1 and 3.6 kcal/mol, respectively).[3]

Furthermore, substitution of EE by AA in octapeptides has been reported to decrease the affinity about 15-fold,[15] which agrees with our results (Table 1). From this we conclude that the data from SPR competition experiments can be used in a thermodynamic analysis. The van't Hoff plots show shallow curvature, which hampers accurate determination of the heat capacity. The results indicate that the heat capacity has a modest value, as was also reported by Bradshaw et al.[16] for octamer peptides binding to $p60^{src}$ SH2 domain. The results of the van't Hoff analysis show that substitution of EE in Ac-pYEEI-NH_2 by AA or GG is unfavorable

Table 1. Thermodynamic parameters for binding of pYEEI-derived peptides to the $p56^{lck}$ SH2 domain at 25 °C as derived from van't Hoff analysis

Compound	K_d (μM)	ΔG° (kcal/mol)	ΔH° (kcal/mol)	$T\Delta S^\circ$ (kcal/mol)	ΔC_p (cal/mol/K)
Ac-pYEEI-NH_2	0.62±0.10	−8.46±0.10	−5.5±0.7	2.9±0.7	−245±206
Ac-pYAAI-NH_2	8.5±1.8	−6.92±0.26	2.1±1.1	8.9±1.1	−141±343
Ac-pYGGI-NH_2	390±67	−4.65±0.10	6.0±1.4	10.7±1.4	501±427

Table 2. Minimum energy conformers from the computational conformation analysis of pYEEI derived peptides (generated as described in Experimental). Included is the energy of the conformers relative to the energy of the minimum energy conformation (ΔE_i) and the number of times that each unique conformer was found

	Ac-pYEEI-NH_2		Ac-pYAAI-NH_2		Ac-pYGGI-NH_2	
	ΔE_i (kcal/mol)	Number of times found	ΔE_i (kcal/mol)	Number of times found	ΔE_i (kcal/mol)	Number of times found
1	0	11	0	35	0	7
2	0.348	6	0.353	19	0.123	6
3	1.16	3	1.07	6	0.446	3
4	1.40	1	1.46	3	0.508	3
5	1.45	1	1.51	3	0.624	2
6	1.49	6	1.64	2	0.695	2
7	1.65	5	1.77	2	0.740	2
8	1.75	3	1.80	2	0.785	2
9	1.78	1	1.85	2	0.830	2
10	1.79	2	2.00	1	0.959	1

for the enthalpy change and favorable for the entropy change upon binding.

To study the peptide conformations in solution and to estimate the contribution of conformational entropy to binding a computational conformation analysis was performed, using the program Generate[12] combined with a Monte-Carlo search with MacroModel.[17] This provides an efficient method for an extensive Monte-Carlo search of the vast conformational hyperspace of peptides. Using this approach the energy minimum of a peptide in solution as well as the distribution of low energy conformations in solution can be derived from which an estimation of the conformational entropy (S_{conf}) was made using the Boltzmann distribution as described in the experimental section. The program Generate generated 500 start conformations of the peptides that effectively cover the conformational hyperspace. These startconformations were subjected to a Monte Carlo search with 5000 steps. The use of these start conformations reduces the risk to explore a limited region of the conformational hyperspace. The 10 lowest energy conformers from the Monte Carlo search are listed in Table 2 with the energy as well as the number of times that each unique conformation was found. This number is an indication for the convergence in the search.[12] The search for all three peptides converged very well as can be seen from Table 2. The distribution of the low energy levels for Ac-pYEEI-NH_2 and Ac-pYAAI-NH_2 is almost the same, which is an indication that these peptides have equal flexibility. The peptide Ac-pYGGI-NH_2 has more low energy levels than the two other peptides, which suggests that this peptide is more flexible. In Figure 4 the four lowest energy conformers are superimposed on C-α of the amino acids. The low energy conformers of Ac-pYEEI-NH_2 are very similar, for Ac-pYAAI-NH_2 there is slightly more difference whereas Ac-pYGGI-NH_2 shows much more difference. In all low energy conformations isoleucine is bent towards the aromatic ring apparently forming a hydrophobic interaction. Finally, the statistical conformational entropy for the peptide in solution (S_{conf}) is estimated (Table 3) from the distribution over the energy minima. Taking the computational results together, we conclude that Ac-pYEEI-NH_2 is not significantly more rigid in solution than Ac-pYAAI-NH_2.

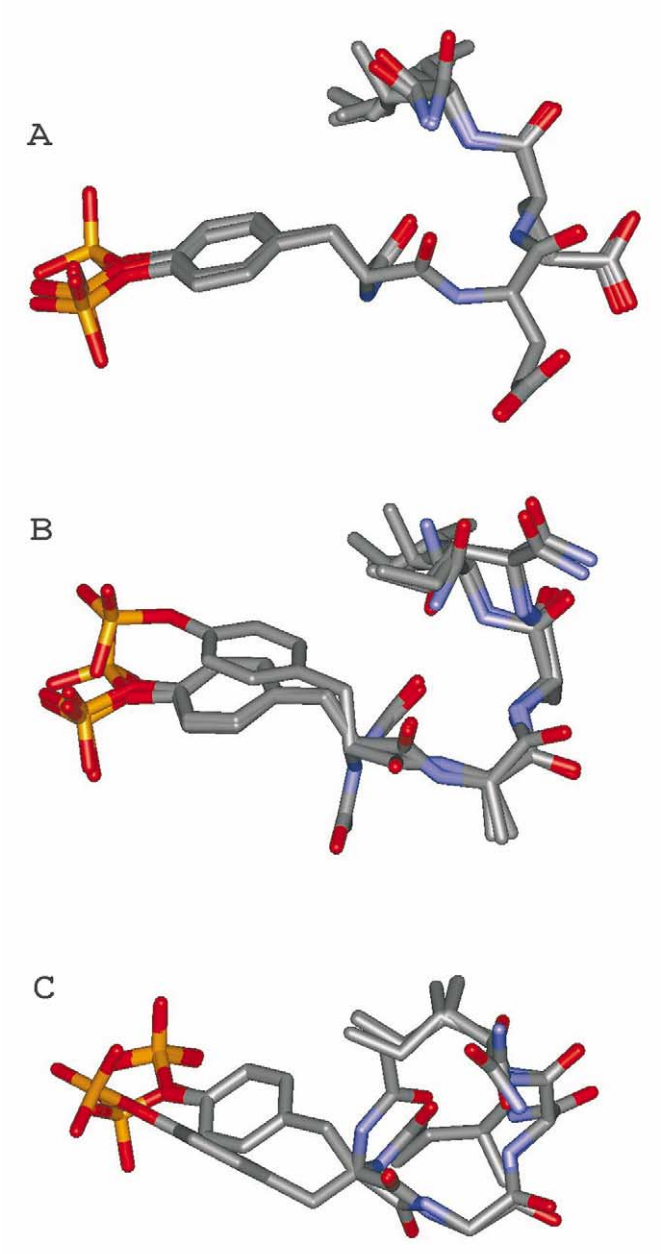

Figure 4. Overlay of the four lowest energy conformers for each peptide found in the conformational search. The structures were overlaid on C-α of the amino acids. (A) Ac-pYEEI-NH_2, (B) Ac-pYAAI-NH_2 and (C) Ac-pYGGI-NH_2.

To compare the solution conformations to the bound conformation, the global minimum energy conformation of Ac-pYEEI-NH_2 has been superimposed on the conformation of the peptide bound to $p56^{lck}$ SH2 domain (Fig. 5). It is striking that the minimum energy conformation of the pYE-moiety is very similar to the bound conformation.

In an attempt to get experimental information on the conformation of the peptides in solution, NMR experiments were performed. In general it is seldom possible to observe distinct conformations in NMR for such small peptides due to their high flexibility. Our experiments were performed at 4 °C to increase the conformational preferences of the peptides. The ROESY spectrum of Ac-pYEEI-NH_2 shows an aromatic ring-isoleucine interaction (Fig. 6A) and an interaction between the pY(NH) and the pY + 1 E(NH) (Fig. 6B). Both interactions are in agreement with the calculated minimum energy conformation, in which the isoleucine is bent towards the aromatic ring. The fact that these

Table 3. Calculated conformational entropy (S_{conf}) in solution as calculated using eq 3 (see Experimental)

Compound	S_{conf} (cal/mol K)
Ac-pYEEI-NH_2	5.5
Ac-pYAAI-NH_2	6.4
Ac-pYGGI-NH_2	10.7

cross peaks can be observed for such a small peptide indicate that indeed predominant conformations in solution resemble the calculated low-energy conformations. The hydrophobic isoleucine-aromatic interaction is also observed in the spectrum of Ac-pYAAI-NH_2, but not in Ac-pYGGI-NH_2 (results not shown).

Discussion

This study was designed to evaluate the role of electrostatically driven conformational control in the binding of pYEEI-peptides to the $p56^{lck}$ SH2 domain. We studied solution conformation and flexibility in relation to binding energetics by combination of a thermodynamic analysis, a computational conformation analysis and NMR. Our hypothesis was that the pYEEI-peptide ligand is preorganized for interaction with the $p56^{lck}$ SH2 domain, as the pYEEI-peptide is expected to have a more or less extended conformation in solution due to electrostatic repulsion between the negative charges of the pYEE-moiety. The peptide Ac-pYEEI-NH_2 was compared to Ac-pYAAI-NH_2 and Ac-pYGGI-NH_2, for which this electrostatic repulsion does not apply. We are of course aware of the fact that such an approach has limitations, as changes in the ligand do not only change flexibility or solution conformation but give also rise to changes in a variety of energetic contributions to binding. Nevertheless, the outlined approach provides more insight into the role of flexibility in the binding of Ac-pYEEI-NH_2 based ligands to the $p56^{lck}$ SH2 domain, which is relevant for drug design.

It is remarkable that the computed conformation of the pYE-moiety at the global minimum in solution agrees with the conformation of the pYEEI-peptide bound to the SH2 domain as represented in Figure 5. Such minimum energy conformation is predominant in solution as was confirmed by NMR, which demonstrates a hydrophobic interaction between the aromatic ring of the phosphotyrosine and the isoleucine residue and a cross peak for the pY and pY + 1 E NH protons (Fig. 6). As a practical implication, conformational restriction of the pYE moiety might not be effective, which has indeed been observed.[2,18]

In contrast to the pYE moiety, the position of the pY + 2 EI moiety in the bound conformation differs considerably from the minimum in solution (Fig. 5). Upon binding it changes from a bent conformation to an extended conformation, which apparently costs energy. Calculations showed that the conformational enthalpy difference between the bound and free peptide is rather large for Ac-pYEEI-NH_2 (14.6 kcal/mol) suggesting that in the bound state unfavorable conformational enthalpy is compensated for by favorable interaction energy between peptide and the SH2 domain. From this point of view constriction of the pY + 2 EI moiety might improve binding.

From the computational conformation analysis no evidence for electrostatic rigidization of the solution conformation can be derived. The computed values for S_{conf} for Ac-pYEEI-NH_2 and Ac-pYAAI-NH_2 are similar (Table 3). Furthermore, the thermodynamic analysis does not indicate a more favorable entropic contribution to binding for Ac-pYEEI-NH_2 (Table 1).

Inspection of Table 1 shows that substituting EE in Ac-pYEEI-NH_2 for AA or GG gives an increased ΔH° value that is counterbalanced by an increased ΔS° value, which suggests that thermodynamics of binding is controlled by entropy-enthalpy compensation. Indeed, plotting ΔH° versus ΔS° demonstrates an entropy-enthalpy compensation relationship for these three compounds, as represented in Figure 7. Remarkably, ITC derived ΔH° and ΔS° values determined by Bradshaw et al.[16,19] and Charifson et al.[3] for binding of various pYEEI derived peptides and peptidomimetics to wild type and mutated $p60^{src}$ SH2 domains, obey the same entropy–enthalpy compensation relationship (Fig. 7). Observed entropy–enthalpy compensation relationships can be artifacts because in thermodynamic analysis statistical errors tend to distribute estimates of the enthalpy and entropy along a straight line, that is characterized by a slope that is equal to the harmonic mean of the experimental temperatures.[20] In our case the

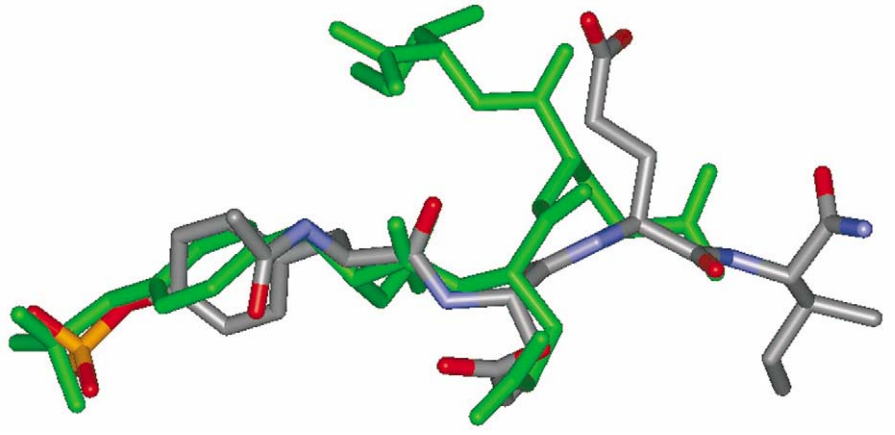

Figure 5. Overlay of the computed unbound minimum energy conformation of Ac-pYEEI-NH_2 (green) and the conformation bound to the $p56^{lck}$ SH2 domain, Protein Data Bank entry 1LCJ (colored by atom type). The overlay was defined by C-α and the charges of pY and pY + 1 E.

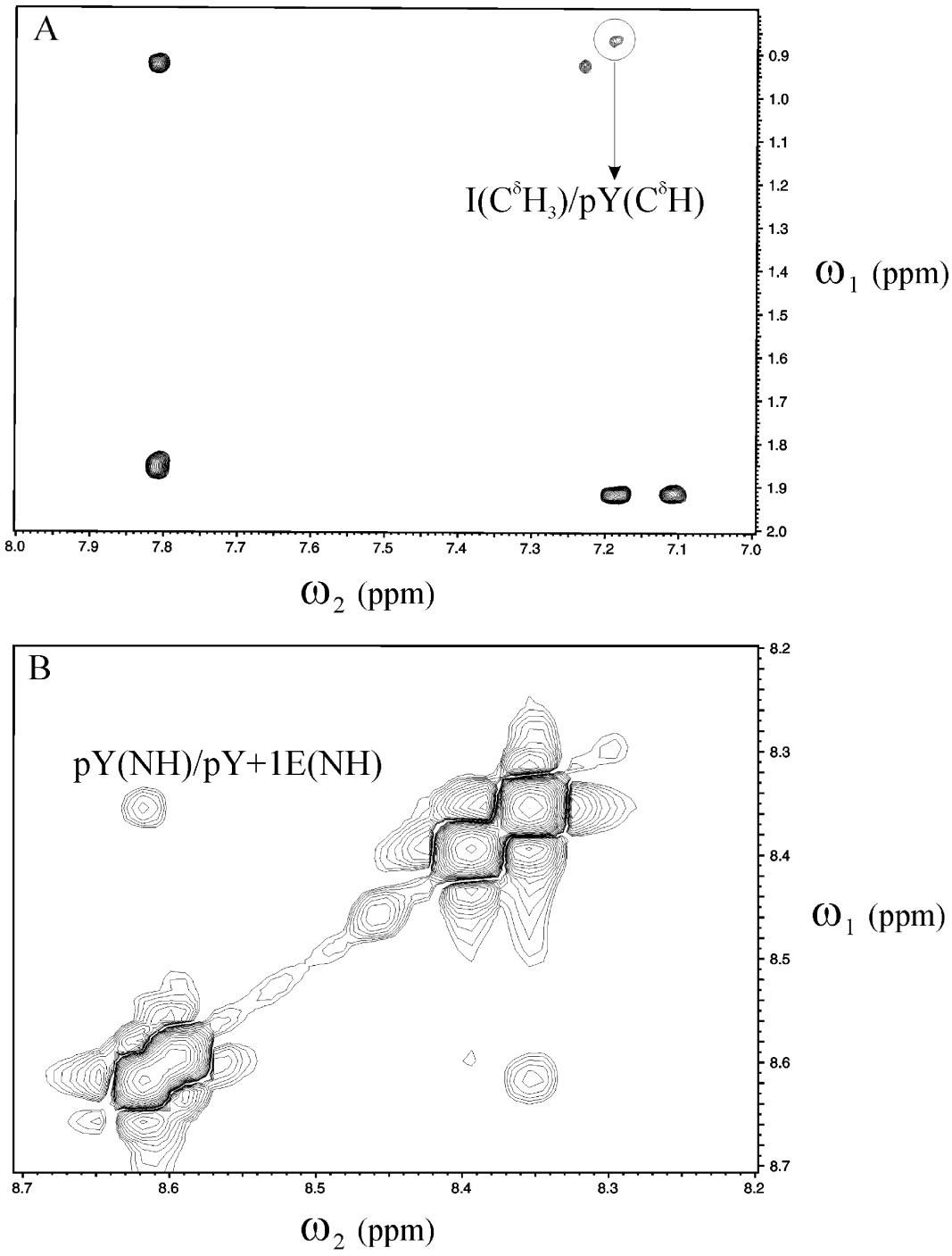

Figure 6. ROESY NMR-spectra. (A) Part of the spectrum of Ac-pYEEI-NH_2 in which the coupling between the aromatic moiety of pY and the aliphatic moiety of I is indicated. (B) part of the spectrum of Ac-pYEEI-NH_2 in which the pY(NH) and pY + 1 E(NH) coupling is indicated.

slope equals 383 K that is significantly deviant from the experimental harmonic mean temperature, which is close to 298 K thus indicating that the observed entropy–enthalpy compensation is a physical phenomenon rather than a statistical artifact. The physical origin of entropy–enthalpy compensation is not fully understood, however it is a general occurring phenomenon in ligand receptor interactions[21–25] for which several explanations have been suggested. Strong interactions with a favorable enthalpy like hydrogen bonding and electrostatic interactions might be counteracted by loss of residual motion and unfavorable entropy changes.[25] The enthalpy–entropy compensation phenomenon has also been attributed to solvent reorganization that accompanies the binding process.[21,26] Water plays in important role in binding of pYEEI ligands to the p60^{src} SH2 domain especially for the E in the pY + 2 position that plays a key role in maintaining a water mediated hydrogen bonding network in the interface of the complex.[11] Henriques and Ladbury[11] suggest that A and G substitutions at pY + 2 result in reduction of the enthalpic contribution and

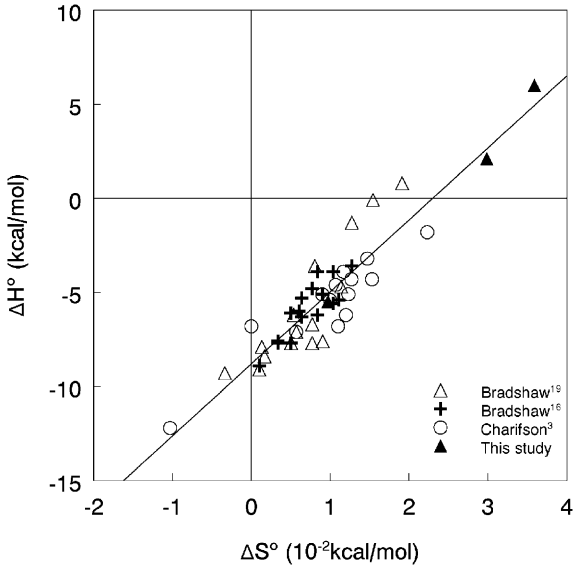

Figure 7. Enthalpy–entropy compensation for binding of pYEEI derived peptides and peptidomimetics to the $p60^{src}$ and $p56^{lck}$ SH2 domains. ▲ = Data from this study, △ = Bradshaw et al.,[19] + = Bradshaw et al.,[16] ○ = Charifson et al.[3]

concomitant increase in entropy by distortion of this water assisted hydrogen bonding network. Compounds with such substitutions are also included in Figure 7 and obey the general entropy–enthalpy compensation relationship. The implication of the observed enthalpy–entropy compensation is that efforts to increase binding enthalpy will be (partly) counteracted by loss of entropy.

Finally, in view of reported inconsistencies between thermodynamic parameters derived from van't Hoff analysis and calorimetry,[27] it is interesting to observe that the SPR and ITC derived parameters obey the same enthalpy–entropy relationship and give comparable parameters for binding of Ac-pYEEI-NH_2 to an SH2 domain. Published thermodynamic studies using SPR are still scarce so we consider the consistency between the SPR and ITC data as presented in Figure 7 as a validation of our SPR based approach. We do not subscribe the view of Cooper[28] that perturbations introduced by immobilization hampers a thermodynamic analysis of SPR derived data, because carefully designed competition experiments can cancel out artifacts due to immobilization.[13,14] In general calorimetry will give more accurate enthalpy- and heat capacity values compared to SPR, however additional buffer and pH effects have to be carefully considered. Furthermore, affinity constants, especially high affinities ($K_a > 10^7$ M^{-1}) can be better assayed by SPR.

Conclusion

Van't Hoff analysis of SPR derived binding constants appeared to be effective in determining enthalpic and entropic contributions to binding of short pYEEI derived peptides to the $p56^{lck}$ SH2 domain. The computational approach yielded low energy conformations in solution in which the isoleucine bends towards the phosphotyrosine apparently due to a hydrophobic interaction, which is also seen by NMR. We found that especially the solution conformation of the pYE moiety agrees with the bound conformation. This is relevant for design of pYEEI mimetics as inhibitors of the $p60^{src}$ and $p56^{lck}$ SH2 domain and suggests that conformational constriction in this part is not needed. Indeed, Davidson et al.[18] and Burke et al.[2] found that rigidization in the pYE moiety does not increase affinity. We found no indications for favorable entropy due to rigidification of the solution conformation by electrostatic repulsion of the four negative charges in the pYEEI-peptide. Instead, we found that the interaction between Ac-pYEEI-NH_2 derived peptides and peptidomimetics and the $p56^{lck}$ SH2 domain is controlled by entropy-enthalpy compensation. The observed enthalpy–entropy compensation indicates that design efforts to increase binding enthalpy will be partly counteracted by loss in entropy.

Experimental

SH2 domain of $p56^{lck}$

The GST fusion protein of the $p56^{lck}$ SH2 domain was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). This protein contains the amino acids 120-226 of the $p56^{lck}$ kinase of the mouse sequence. The molecular weight of the SH2-GST fusion protein is 39,000 Da.

Peptide synthesis

The peptides were synthesized by an Applied biosystems 433A Peptide synthesizer using the Fastmoc protocol on 0.25 mmol Rink Amide resin (load 0.33 mmol/g). Commercially available Fmoc-amino acid building blocks were used including Fmoc-Tyr(*t*Bu)–OH, Fmoc-Glu(O*t*Bu)–OH, Fmoc-Gln(Trt)–OH, Fmoc-Ile–OH, Fmoc-Pro–OH, Fmoc-Leu–OH (Advanced Chemtech, Louisville KY), Fmoc-εAhx–OH (Fmoc-6-aminohexanoic acid) and Fmoc-Tyr(PO(OBzl)OH)–OH (Nova Biochem, Bad Soden, Germany). The amino acids and the coupling reagents 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/1-hydroxybenzotriazole (HOBt) were used in fourfold and *N,N*-diisopropylethylamine (DiPEA) in a 12-fold excess. 1-Methyl-2-pyrrolidinone (NMP) was used as solvent and cleavage of the Fmoc group was performed with 20% piperidine in NMP. Final acetylation of the N-terminus was performed with a mixture of acetic anhydride, DiPEA and HOBt in NMP (0.5 M, 0.125 M and 1.5 mM, respectively). Cleavage from the resin and deprotection was performed by a mixture of 9.5 mL trifluoroacetic acid (TFA), 125 μL 1,2-ethanedithiol (EDT), 125 μL triisopropylsilane (TiS) and 250 μL H_2O (3 h). The peptides were precipitated in ice-cold methyltertbutylether (MTBE)/hexane (1/1), purified by preparative HPLC and lyophilized. For

preparative HPLC an Adsorbosphere C_8 preparative column was used, with as eluent a gradient of 0–95% acetonitrile/water (v/v) and 0.1% TFA after which the purity was checked by analytical HPLC (C_{18}90Å5U). The identity of the peptides was confirmed by mass spectrometry and 500 MHz ^{1}H NMR analysis.

Surface plasmon resonance (SPR) measurements

Experiments were performed on a double channel IBIS II SPR instrument (IBIS Technologies, Enschede, The Netherlands) that was equipped with a CM5 sensor chip (BIAcore AB, Uppsala, Sweden). These chips contain a carboxymethylated dextran surface to which the primary amine of Ahx in the peptide Ahx- EPQpYEEI-PIYL-NH_2 can be coupled with *N*-hydroxysuccinimide (NHS)/ *N*-ethyl-*N*′-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC) chemistry using the Amine Coupling Kit (BIAcore AB, Uppsala, Sweden). The sensor surface of the sample cell was activated with EDC/NHS for 5 min, after which 2 mM of the Ahx-peptide in 100 mM borate buffer with 1 M NaCl (pH 8.3) was coupled to the chip for 10 min. After that the chip was deactivated by 1 M ethanolamine (pH 8.5) for 7 min. The reference cell was treated identically except that no peptide was added. The net SPR signal was obtained by subtracting the signal in the reference cell from that in the sample cell. In a typical experiment 35 μL of a sample in running buffer was added by an autosampler into the sample cell as well as to the reference cell. The running buffer was 10 mM Hepes, 3.4 mM EDTA, 150 mM NaCl and 0.005% Tween-20, pH was titrated with NaOH to 7.4. The chip was regenerated with 0.2% SDS in 50 mM HCl. Competition experiments were performed by adding to the cells 50 nM of SH2 domain premixed with various concentrations of phosphopeptide at temperatures ranging from 10 to 40 °C.

Thermodynamic analysis

Thermodynamic binding constants (K_d) for the interaction of peptides with p56^{lck} SH2 domain have been assayed based on the method of Morelock et al.,[14] as described previously.[13] A correction was made for depletion of analyte from the bulk solution in the cuvette due to binding to the sensor.[29] The temperature effect on the intrinsic SPR signal was included in this correction. From the affinity data at various temperatures van't Hoff plots ($\ln K_a$ vs $1/T$) were constructed and the data fitted to the integrated van't Hoff equation (Eq 1). This yielded the entropy change ΔS° and the enthalpy change ΔH° at the reference temperature upon binding, as well as the heat capacity change ΔC_p, which was assumed to be independent of temperature.

$$\ln K_A = \frac{-\Delta H^\circ(T^\circ)}{RT} + \frac{\Delta S^\circ(T^\circ)}{R} + \frac{\Delta C_p}{R}.\left[\left(\frac{T-T^\circ}{T}\right) - \ln\left(\frac{T}{T^\circ}\right)\right] \quad (1)$$

In this equation K_a is the association constant in solution, T the temperature, T° the reference temperature of 25 °C (298.15 K) and R the gas constant.

Computational conformation analysis

A conformational search was performed according to the method recently published by Bultinck et al.,[12] which in brief comprises two steps. First 500 conformations of the peptide are constructed from building blocks of di- and tripeptide conformational libraries representative of each type of amino acid, including phosphotyrosine, using the Generate program. These different starting conformations are used to initiate a Monte Carlo conformational search with 5000 steps. The use of 500 different starting structures strongly reduces the risk of exploring only a limited region of the potential energy hyperspace. All energy calculations and geometry optimizations were performed using the MacroModel program,[17] using the MMFF force field,[30] and implicit water solvation using the GB/SA model.[31] Only those conformers with a relative energy below 25 kcal/mol are retained.

An expression for the conformational entropy in solution is derived from the population of each energy level. The fraction of the molecules in an energy level i (P_i) can be calculated from the Boltzmann distribution (Eq 2). In this equation is ΔE_i the energy of the minimum i minus the energy of the global minimum and j is the number of energy levels considered. The total energy of the conformers is used as calculated by MacroModel.

$$P_i = \frac{e^{\frac{-\Delta E_i}{RT}}}{\sum_j e^{\frac{-\Delta E_i}{RT}}} \quad (2)$$

Subsequently, the statistical conformational entropy (S_{conf}) for the peptides was calculated with the Boltzmann formula for entropy, Eq 3.

$$S_{conf} = -R\sum_j P_i.\ln P_i \quad (3)$$

NMR experiments

All NMR experiments were performed on samples containing 4 mM peptide dissolved in 9/1 H_2O/D_2O (v/v) buffered with 20 mM sodium phosphate at pH 7.0. Dioxane, which resonates at 3.75 ppm relative to trimethylsilylpropanesulphuric acid, was added as an internal standard. ^{1}H NMR spectra of the peptides were recorded at 278 K on a Varian Inova 500 MHz spectrometer. The ^{1}H resonances were assigned using standard methods. Conformational information was derived from ROESY experiments (τ_m = 500 ms). A spectral width of 5006.3 Hz was used for all spectra. Water suppression was accomplished using the WATERGATE suppression method[32] and data were processed using the program NMRPipe.[33]

Acknowledgements

We thank Professor Dr. Jan P. A. E Tolleneare for helpful advice and discussion.

Appendix. Supplementary Material

NMR shift data and assignments

Peptide	NH	$C^{\alpha}H$	$C^{\beta}H$	$C^{\gamma}H$	$C^{\delta}H$	Other
Ac-pYEEI-NH_2						
pTyr	8.35	4.56	2.87, 3.15		7.19	7.11 ($C^{\varepsilon}H$), 1.92(Acetyl)
Glu	8.62	4.27	1.92, 2.02	2.25		
Glu	8.60	4.30	1.95, 2.04	2.27		
Ile	8.39	4.11	1.85	1.21, 1.51	0.86	0.93($C^{\gamma}H_3$), 7.23, 7.81 (NH_2)
Ac-pYAAI-NH_2						
pTyr	8.30	4.53	2.94, 3.09		7.18	7.12 ($C^{\varepsilon}H$), 1.94(Acetyl)
Ala	8.39	4.27	1.33			
Ala	8.36	4.31	1.38			
Ile	8.30	4.09	1.86	1.23, 1.51	0.88	0.95($C^{\gamma}H_3$), 7.25, 7.78 (NH_2)
Ac-pYGGI-NH_2						
pTyr	8.49	4.51	3.00, 3.08		7.18	7.12 ($C^{\varepsilon}H$), 1.96(Acetyl)
Gly	8.67	3.83, 3.92				
Gly	8.17	3.96				
Ile	8.13	4.16	1.90	1.21, 1.47	0.87	0.94($C^{\gamma}H_3$), 7.26, 7.84 (NH_2)

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