$CHCl_3$/MeOH) and recrystallized from $CHCl_3/C_6H_{14}$ to give **75**: mp 272-273 °C; NMR ($CDCl_3$) δ 2.08 (4 H, m), 3.05 (2 H, t), 4.16 (2 H, t), 5.56 (2 H, br s), 8.32 (1 H, s). Anal. ($C_9H_{11}N_5$) C, H, N.

Method E. 8,9-Di-*n*-pentyladenine (74). To a solution of **36** (500 mg, 2.44 mmol) in dry *N*-methylpyrrolidinone (1.5 mL) was added NaH (50%, 117 mg, 2.44 mmol), and the mixture was stirred for 10 min. Next, 1-bromopentane (368 mg, 2.44 mmol) was added dropwise with stirring over 0.5 h, and the reaction mixture was treated with H_2O, extracted with EtOAc, dried ($MgSO_4$), and evaporated to dryness. Purification by chromatography (SiO_2, CH_2Cl_2/MeOH) gave a crude product which was recrystallized from $CHCl_3/C_6H_{14}$ to give **74** (272 mg, 47%): mp 111-112 °C; NMR ($CDCl_3$) δ 0.91 (6 H, m), 1.37 (8 H, m), 1.82 (4 H, m), 2.83 (2 H, t), 4.12 (2 H, t). 5.64 (2 H, br s), 8.31 (1 H, s). Anal. ($C_{15}H_{25}N_5$) C, H, N.

Method F. 6,8-Diaminopurine (32). A solution of **31** free base (1.00 g, 4.67 mmol) in excess benzylamine (5 mL) was heated at 150 °C with stirring for 2 h. The resulting orange solution was poured into $CHCl_3$ (100 mL) to precipitate **33** free base as a fine, white solid (0.84 g, 75%). A sample of this product was treated with 1 N HCl in excess to give the crude hydrochloride, and recrystallized from H_2O to give **33**, mp 198-200 °C.

The free base of **33** (725 mg, 3.02 mmol) was dissolved in liquid NH_3 (250 mL) and small pieces of Na (2.84 g, 123 g-atom) were added over 3 h, with stirring until the color was no longer discharged. Excess NH_4Cl was added and the NH_3 was allowed to evaporate. The residue was chromatographed (SiO_2, $CHCl_3$/MeOH/NH_4OH 10:5:1) to give a yellow solid which was extracted into boiling EtOH, treated with activated charcoal, filtered, and evaporated to give a crude product (180 mg, 40%). This was dissolved in 1 N KOH and adjusted to pH 5 with 1 N HCl, filtered and cooled to give **32** as a white powder: mp >300 °C; NMR (DMSO-d_6) δ 6.51 (2 H, br s), 6.72 (2 H, br s), 7.96 (1 H, s). Anal. ($C_5H_6N_6$·2HCl) C, H, N.

PI 4-Kinase Assay. Highly purified human erythrocyte plasma membranes were prepared by the method described by Hawkins et al.[29] Assays were carried out in a 200 μL final volume containing membranes (0.36 mg of membrane protein), PI (0.12 mM, 0.04 μCi [2-^{3}H]PI), ATP (100 μM), ouabain (100 μM), sodium metavanadate (100 μM), dithiothreitol (5 mM), Triton X-100 (0.6%), KCl (120 mM), NaCl (30 mM), EGTA (1 mM), $MgCl_2$ (5 mM), HEPES (50 mM, pH 7.4), plus inhibitor or vehicle. Mixtures were incubated for 10 min at 30 °C, and the reaction was terminated by the addition of 0.75 mL of $CHCl_3$/MeOH/concentrated HCl (40:80:1). Addition of 0.25 mL of $CHCl_3$ and 0.25 mL of 0.1 M HCl gave two phases, and the organic phase, containing the phospholipids, was separated, and made up to 1 mL with $CHCl_3$, to which 0.2 mL MeOH and 0.2 mL of 1 M NaOH in 5% v/v aqueous MeOH was added to initiate deacylation. After 20 min at room temperature 1 mL of $CHCl_3$, 0.6 mL of MeOH, and 0.6 mL of H_2O were added to the samples and they were mixed vigorously. The two phases were separated by centrifugation, and 1 mL of the upper aqueous phase containing the deacylated phospholipids was removed. This was made approximately neutral with 0.8 mL 1 M H_3BO_3 and brought up to 5 mL with $Na_2B_4O_7$ (5 mM).

Samples were loaded onto Dowex AG1-X8 columns (200-400 mesh, formate form). Next, 20 mL of 0.005 M $Na_2B_4O_7$/0.18 M HCO_2NH_4 was applied to the columns to leave 1-(*sn*-glycer-3-ylphosphoryl)-D-*myo*-inositol 4-phosphate and 1-(*sn*-glycer-3-ylphosphoryl)-D-*myo*-inositol 4,5-bisphosphate as the only deacylated ^{3}H-labeled phospholipids remaining on the columns. The former compound was specifically eluted with 10 mL 0.3 M HCO_2NH_4/0.1 M HCO_2H and 10 mL of Triton X-100/xylene scintillant was added prior to counting for ^{3}H radioactivity.

Acknowledgment. We gratefully acknowledge the contributions of the Physical Organic Chemistry Department in providing analytical data and Drs. C. P. Downes and C. H. Macphee for valuable discussions.

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Thermodynamics of the Interaction of Inhibitors with the Binding Site of Recombinant Human Renin

D. E. Epps,*,† J. Cheney,‡ H. Schostarez,§ T. K. Sawyer,∥ M. Prairie,† W. C. Krueger,† and F. Mandel⊥

Physical and Analytical Chemistry Research, Hair Growth Research, Biopolymers Research, and Cardiovascular Diseases Research, The Upjohn Company, 7000 Portage Road, Kalamazoo, Michigan 49001, and Department of Chemistry, Harvard University, Boston, Massachusetts. *Received December 6, 1989*

The independent subsite model is widely used for the design of peptide inhibitors of enzymes with extended active sites. This model assumes that the subsites are independent of each other and that the free energies of binding contributed by the several subsites are additive. We questioned the strict application of this model for structure–activity studies, since one can, a priori, conceive of likely deviations from this model. Accordingly, we tested the independent subsite model by measuring the thermodynamic binding parameters of a series of peptide inhibitors of human renin. This enzyme–inhibitor system was chosen as a model by virtue of the high degree of specificity of renin for its natural substrate, angiotensinogen, and the availability of a large number of structurally similar peptide inhibitors. Although we found the general mode of binding of these renin inhibitors to be primarily hydrophobic, serious deviations from additivity and independent subsite model constraints were observed. We conclude that an important determinant of binding is most probably the conformation assumed by the peptide inhibitor in solution. Thus, we suggest that caution be exercised in using affinity constants to assess the interactions of peptide inhibitors with human renin and possibly with other enzymes having extended binding sites. Furthermore, the thermodynamic parameters of a class of compounds provide more information as to the mode of binding of ligands to their respective receptors than do dissociation constants.

Introduction

Traditionally, the interaction of substrates and inhibitors with the active site of enzymes and receptors is probed by measuring the changes produced upon varying the chemical structure of the ligand. This approach proved to be particularly fruitful in establishing the binding properties, and hence the specificity, of endopeptidases. These enzymes often possess an extended binding site ideally suited for positioning an intended cleavage site through recognition of several amino acid residues preceding and following the scissile bond. The results of investigations on protease specificity, particularly those concerning the in-

† Physical and Analytical Chemistry Research, The Upjohn Co.
‡ Department of Chemistry, Harvard University.
§ Hair Growth Research, The Upjohn Co.
∥ Biopolymers Research, The Upjohn Co.
⊥ Cardiovascular Diseases Research, The Upjohn Co.

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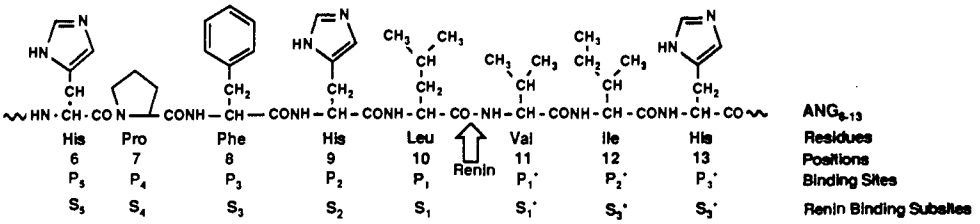

Figure 1. Schematic representation of the extended renin binding site, showing the interactions of the individual substrate amino acid residues with corresponding active-site counterparts. The numbering of the active-site residues is as described previously by Schechter and Berger.[1]

teraction of papain with oligopeptides, led to a general model[1] for peptide ligand–enzyme interactions. This model is widely used[2-4] for the rational design of peptide drugs acting as inhibitors or substrates for a variety of enzymes of pharmacological significance. As applied to drug design, the use of the model often implies that the extended binding site is composed of rigid and independent subsites. Interaction of the peptide ligand with the enzyme active site is optimal when each of the peptide's amino acid moieties occupies one subsite specific for that amino acid. The model further implies that the interactions of the ligand amino acid moieties with their respective binding subsites are independent of one another. Thus, the experimentally observed affinity constant should be resolvable into independent factors, each measuring the contribution of one particular binding interaction. According to this reasoning, a structural change in one amino acid of the ligand should modify the interaction of the ligand with one, and only one, subsite; hence, the accompanying change in affinity should reflect the binding characteristics of that subsite alone. The success of this model in designing protease inhibitor peptides confirmed the validity of the model, at least in semiquantitative terms. A priori considerations suggest, however, that the application of this model for designing drugs may be somewhat oversimplified. It is likely that some minor conformational changes of the protein will accompany the binding of the ligand to the active site. Mathematical models of the dynamics of protein conformation indicate that some cooperativity should inevitably occur between contiguous subsites. Also, the affinity is measured as the free energy difference between the free, solvated ligand and the free, solvated active site on the one hand and the complex on the other hand. If the free ligand is not a random coil, then its secondary structure is the result of interactions between its several amino acid subunits. Because of this cooperativity between the subunits of the ligand, a structural variation within the ligand will influence not only the interaction between the ligand and the active site but also the cooperativity within the ligand. In other words, the changes in affinity will reflect not only changes in ligand–active site interactions but also changes in the solution conformation of the ligand. As a consequence, there should be deviations from additivity because solution conformation is governed by different parameters from those governing enzyme–substrate interactions, and the deviations from additivity should be the greatest for long peptides.

In the light of these possible limitations, we proposed to explore the limits of validity of the use of affinity constants for determining the characteristics of the subsite model. For this purpose we determined the thermodynamic parameters of binding of a series of competitive inhibitors to an enzyme with an extended binding site. If, and only if, the subsite model is truly valid should one observe strict additivity of the individual thermodynamic parameters (Δh, Δs, and Δg) contributed by each amino acid moiety. We have chosen recombinant human renin since it was shown to have an unusually extended binding site and high specificity among proteases[5] and because we had at our disposal a large number of peptide analogue renin inhibitors synthesized as antihypertensive candidates. Furthermore, we recently developed a procedure for the facile measurement of dissociation constants (K_d's) of renin inhibitors.[6]

Renin is an important enzyme in the regulation of blood pressure and is currently being targeted here and elsewhere in the design of new therapeutic agents for the treatment of hypertension. The binding of inhibitors to human renin has been the subject of numerous structure–activity studies.[7-9] The nature of the renin active site confers a high degree of specificity upon the enzyme, both in terms of substrate catalysis and inhibitor binding. The renin active site cleft can accommodate up to eight amino acid residues which correspond to positions 6–13 of the natural substrate angiotensinogen.[10] The catalytic aspartates cleave between the Leu-Val residues designated as the P_1–P_1' sites in the subsite model (Figure 1). Although structure–activity relationships for several series of renin inhibitory peptides have been proposed, no thermodynamic study of the binding of these inhibitors has been undertaken. In the present paper, we propose to show that the strict additivity, which would be required in order to be able to use the affinity constants for determining the properties of subsites, is not observed in the case of the binding of peptide inhibitors to human renin.

Experimental Section

Peptides studied in this report, shown in Figure 2, were syn-

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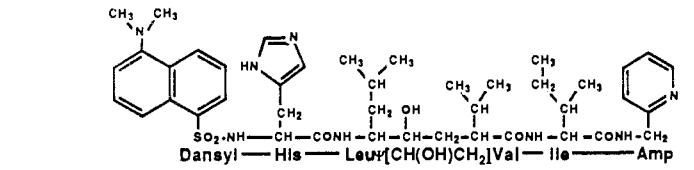

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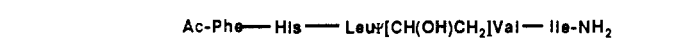

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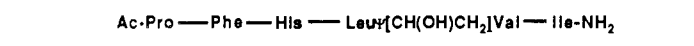

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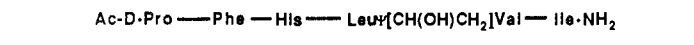

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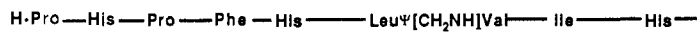

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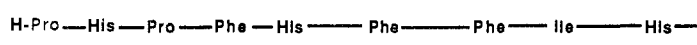

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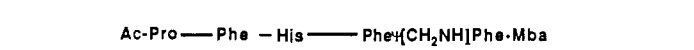

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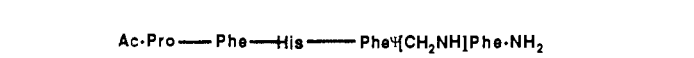

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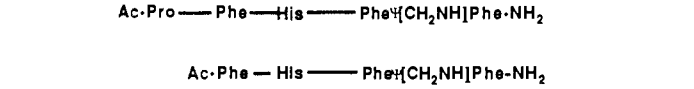

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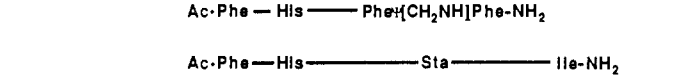
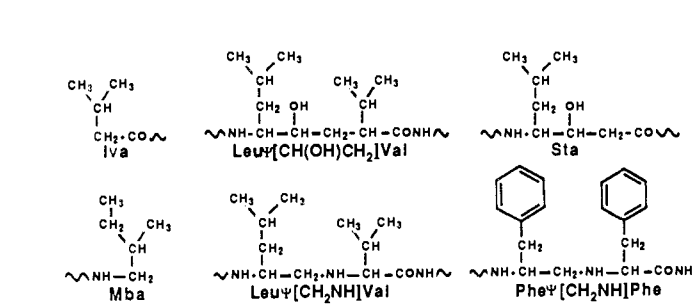


U-72407E

U-72408E

U-72409E

U-77455E



SPECIFIC CHEMICAL MODIFICATIONS OF ANG-BASED RENIN INHIBITORS

Figure 2. Chemical structures of all renin inhibitory peptides are shown in this figure grouped by class based on their putative transition-state isosteres at the P_1–P_1' positions: panel A, Leu-Val alcohol compounds; panel B, reduced Phe-Phe inhibitors; Panel C, statine compounds.

thesized by solution- or solid-phase procedures and purified to homogeneity by HPLC. All peptide structures were ascertained by fast atom bombardment mass spectrometry and amino acid analysis and detailed synthetic procedures for the dansylated peptide U-80215E were given previously.[6] The inhibitors were chosen on the basis of their structural diffrences and their wide range of affinities, and the concentrations of stock solutions of the peptides were determined by amino acid analysis. Recombinant human renin was isolated and purified as described previously[11] and the concentration of renin stock solutions also determined by amino acid analysis. The dissociation constants (K_d's) were determined over a temperature range of 8–37 °C by the fluorescence displacement assay[6] using a Perkin-Elmer MPF66 spectrofluorometer in the ratio mode. The temperature was maintained with a Lauda circulating-water temperature bath. Dissociation constants were calculated from experimentally determined fractions bound by using the following equation[6]

$$K_H = \{(H_{total}(K_L/[L]))/(1/f^* - 1 - (K_L/[L]))\} - R_{total}f^*(K_L[L]) \quad (1)$$

where K_H is the dissociation constant of the unlabeled inhibitor, H_{total} is the molar concentration of unlabeled inhibitor, K_L is the dissociation constant of the fluorescent inhibitor (U80215E), [L] is the concentration of fluorescent inhibitor, f^* is the fraction of fluorescent inhibitor bound, and R_{total} is the enzyme (renin) concentration.

Duplicate assays at two different concentrations of inhibitors were performed, and van't Hoff plots of the temperature dependence of the K_d's were constructed from the data.

Circular Dichroism and Ultraviolet Spectroscopy. Ultraviolet absorption measurements were made with a Perkin-Elmer Lambda 7 spectrophotometer calibrated with potassium dichromate solutions, NBS-certified optical density filters, and a holmium oxide filter. The CD spectra of U-71909E and U-77451E were obtained at room temperature (20–22 °C) in 1-cm quartz cuvettes on a JASCO 600 spectropolarimeter calibrated with D-10-camphorsulfonic acid.[12] The buffer was 0.01 M sodium phosphate, pH 7.23. For the studies at 4 °C and 75 °C, the temperature was maintained with a circulating-water bath.

The CD intensity, expressed as a mean residue ellipticity, [θ], in degrees per square centimeter per decimole, was calculated from the equation

$$[J] = R \times (M/C) \times L \times 100 \quad (2)$$

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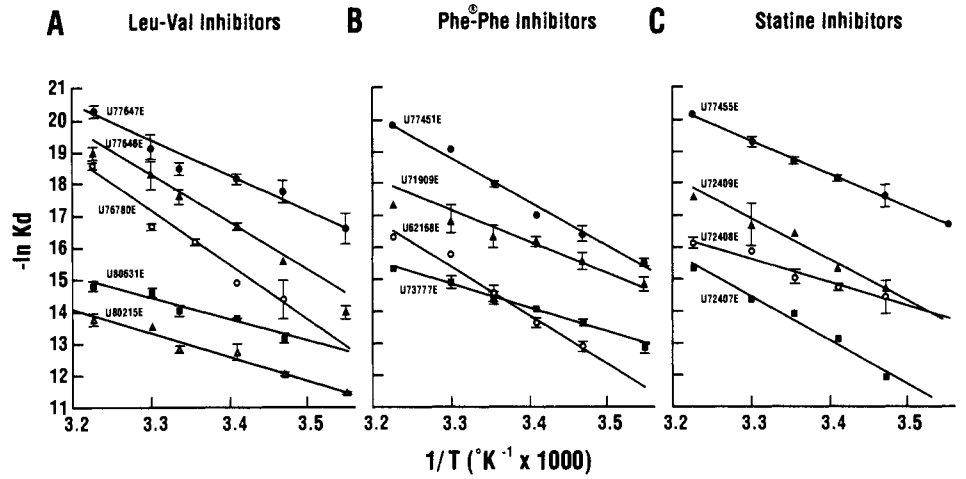

Figure 3. The temperature dependence of the K_d's of the renin inhibitory peptides shown in Figure 1 is shown and determined in the temperature range of 8–37 °C. The K_d's were assessed by using the fluorescence displacement assay[6] as described in the Experimental Section.

where R is the ellipticity measured in degrees; M is the mean residue molecular weight, C is the concentration (g/L), and L is the cell path length in decimeters. The concentrations of the peptide solutions were determined from their respective UV absorbance spectra by using the base-line technique in the 260-nm absorbance region. In this technique, a base line is drawn from wing to wing of the aromatic absorption band. The absorbance is then defined as the distance between the band maximum and the base line of the A (257-nm) band. Using the above method, phenylalanine was determined to have a molar absorptivity of 173 at 257 nm. The concentration of the peptides as determined from their absorption spectra were [U-71909E] = 1.35×10^{-5} M and [U-77451E] = 0.44×10^{-5} M. The mean residue molar concentration would then be $3 \times 1.35 \times 10^{-5}$ M, since there are three "normal" amino acids in the peptide. The molar absorptivity of the peptides in question would then be 3×173 since they each have three Phe groups.

Results

The structures of the renin inhibitory peptides used in this study are given in Figure 2. Several types of inhibitors were chosen, by reason of the similarity of their structure to that of angiotensinogen (ANG) and by virtue of their wide range of affinities. They contain either Leu-Val-OH (reduced Leu-Val) or statine or reduced Phe-Phe, all putative transition-state isosteres at the position corresponding to the angiotensinogen cleavage site (shown in Figure 1). Some of the compounds (e.g., U-71909E and U-77451E) differed by only one modification at a given position in the sequence. Thus, a comparison of the K_d's and thermodynamic binding parameters of these inhibitors should provide insight into the factors associated with inhibitor binding to human renin and the validity of the concept of independent binding subsites as applied to this enzyme.

All inhibitors used in this study had been found to be purely competitive.[6] In other words, their binding is fully reversible and is governed by a single thermodynamic equilibrium constant, according to the scheme

$$E + I \overset{K_d}{\leftrightarrow} EI \tag{3}$$

where

$$K_d = [E][I]/[EI] \tag{4}$$

and [E], [I], and [EI] are the concentrations of the free enzyme, free inhibitor, and the enzyme–inhibitor complex, respectively. The free energy change, $\Delta G°$, corresponding to the dissociation of the enzyme–inhibitor complex, is given by

$$\Delta G° = \Delta H° - T\Delta S° = -RT \ln K_d \tag{5}$$

where R is the gas constant and T is the absolute temperature. The value of K_d was determined at several temperatures ranging from 8 to 37 °C. When van't Hoff plots (Figure 3) were constructed with these K_d values, the experimental data for each individual inhibitor yielded a plot indistinguishable from a straight line ($r > 0.95$). Thus, for the temperature range studied, the $\Delta H°$ of the equilibrium is independent of temperature within our experimental error. The values for $\Delta H°$ and $\Delta S°$ were calculated from the slope and intercept of the plots and the results are given in Table I together with the K_d's at 37 °C. The $\Delta H°$ and $\Delta S°$ values varied over a considerable range, whereas the variance of $\Delta G_{37}°$ was small relative to the changes in the former two parameters. The value of $\Delta G°$ did not correlate either with $\Delta H°$ (Figure 4) or with $\Delta S°$ (data not shown), in toto or within individual sets of inhibitors, indicating that neither $\Delta H°$ nor $\Delta S°$ is dominant in defining the value of $\Delta G°$. In contrast, an excellent

Table I. Thermodynamic[a] and Binding Parameters of Renin Inhibitory Peptides

compd	K_{d37},[b] μM	$-\Delta H°$, kcal/mol	$-\Delta S°$, entropy units	$\Delta G_{37}°$, kcal/mol
		[Leu-Val-OH]		
U-80215E	1 ± 0.02	14.45 ± 0.7	74.2 ± 2.3	8.6 ± 0.1
U-80631E	0.37 ± 0.06	14.28 ± 0.8	75.7 ± 2.7	9.2 ± .06
U-77646E	0.0054 ± 0.0013	28.75 ± 0.6	131.1 ± 2.0	11.5 ± 0.1
U-77647E	0.0013 ± 0.0002	20.33 ± 0.5	105.5 ± 1.6	12.4 ± 0.2
		[PheΨ[CH_2NH]Phe]		
U-73777E	0.22 ± 0.01	14.2 ± 0.3	76.3 ± 0.8	9.4 ± 0.1
U-77451E	0.0025 ± 0.0015	26.7 ± 0.4	125.3 ± 1.4	12.2 ± 0.1
U-71909E	0.029 ± 0.22	13.7 ± 0.5	78.4 ± 2.2	10.6 ± 0.1
		[Statine]		
U-72407E	0.204 ± 0.0012	26.1 ± 0.8	114.8 ± 2.7	9.5 ± 0.1
U-72408E	0.098 ± 0.013	14.69 ± 0.9	79.6 ± 3.2	9.9 ± 0.1
U-72409E	0.023 ± 0.0001	22.63 ± 0.5	108 ± 1.8	10.8 ± 0.2
U-77455E	0.0017 ± 0.0001	21.36 ± 0.2	108.9 ± 0.7	12.4 ± 0.04
		[Phe-Phe]		
U-62168E	0.81 ± 0.019	29.35 ± 0.7	127.4 ± 2.4	8.6 ± 0.2
		[LeuΨ[CH_2NH]Val]		
U-76780E	0.008 ± 0.0012	33.56 ± 0.9	144.3 ± 5.9	11.2 ± 0.4

[a] Calculated from the slope and intercepts of the van't Hoff plots. [b] Mean ± SEM of at least four determinations. [c] Calculated from $\Delta G_{37}° = \Delta H - T\Delta S$ with the values above.

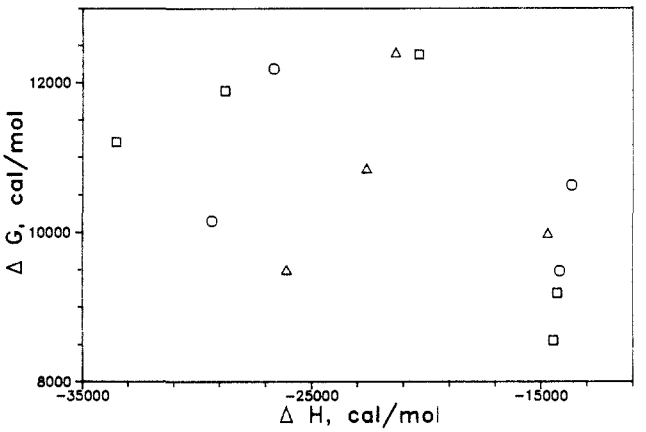

Figure 4. Relationship between ΔH and ΔG of renin inhibitory compounds. Enthalpy and free energy of dissociation values of renin inhibitory peptides were determined as described in the Experimental Section and Table I: □, Leu-Val alcohol compounds; ○, reduced Phe-Phe compounds; △, statine compounds.

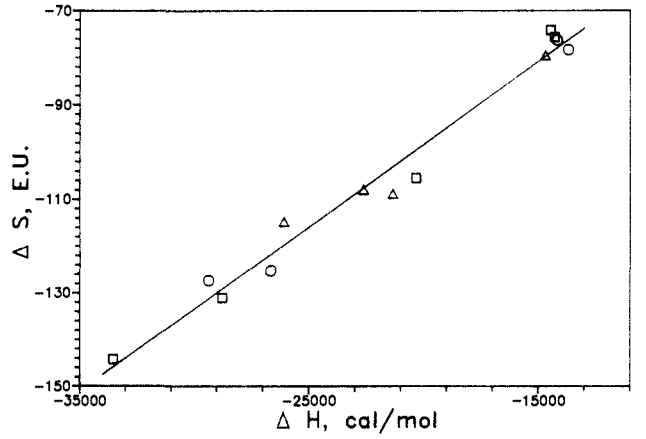

Figure 5. Relationship between ΔH and ΔS of renin inhibitory peptides. Enthalpy and entropy values for all renin inhibitory peptides were determined as described in the Experimental Section and Table I: □, Leu-Val alcohol compounds; ○, reduced Phe-Phe compounds; △, statine compounds.

correlation appeared between the values of $\Delta H°$ and $\Delta S°$ as shown in Figure 5. The slope of the latter plot (r = 0.988 from linear regression) was calculated to be 278 ± 12 K.

If the peptides in question were to exist as folded rather than random structures in solution, then differences in the types of folded structure would contribute to the changes in the thermodynamic binding parameters of the inhibitors. Although it is generally not possible to determine, with certainty, the exact structure of small linear peptides in solution, one can, nevertheless, decide by CD methods whether the peptide is fully in a random structure conformation or not. We have evaluated the molar ellipticity CD curves of two related renin inhibitor peptides (U-71909E and U-77451E, Figure 6, parts A and B). As shown in Figure 6A, in aqueous buffer at room temperature, the CD profile of these two peptides is the same within experimental error. Most importantly, the CD intensity is a function of temperature as illustrated in Figure 6B, with the largest molar ellipticity being observed at 75 °C. Both peptides contain three aromatic rings (Phe), and since aromatic contributions to the CD affect the analyses, we have subtracted the phenylalanine contribution from the CD curves shown in both figures. The correction was performed by subtracting the Phe contribution as determined from the spectrum of the dipeptide Lys-Phe given by Brahms and Brahms.[13] When this large correction for the Phe contribution was made, more CD structure was obtained than for the uncorrected spectra. Since these are small linear peptides, we felt that this correction would unfairly bias the data. Thus the CD spectra shown in Figure 6 are uncorrected. The uncorrected spectra do indeed show that the preferential conformation of these two peptides in solution is not that of a random structure. Given the similarity of all peptides shown in Figure 1, it is likely that this conclusion is valid for the other peptides also.

Discussion

From our measurements of K_d at different temperatures, we evaluated the thermodynamic parameters $\Delta G°$, $\Delta H°$, and $\Delta S°$ for a number of renin inhibitory peptides. The free energy of dissociation measures the difference in energy between the enzyme–inhibitor complex on the one hand, and the solvated inhibitor and the solvated active site on the other hand. According to the subsite model, this free energy is the sum of the partial free energies contributed by the independent interaction of each of the side chains with its corresponding subsite:

$$\Delta G° = \sum \Delta g_i = \sum \Delta h_i - T \sum \Delta s_i \qquad (6)$$

where Δg_i, Δh_i, and Δs_i are the partial values of the thermodynamic functions contributed by the interaction at each subsite. If the partial functions are indeed independent of each other, then replacement of a single side chain by another with a given peptide would result in a free energy change due to the replacement of that partial function only: $\Delta\Delta G = -RT \ln (K_d'/K_d) = \Delta g_i' - \Delta g_i$. In other words, the change should be independent of the rest of the structure of the peptide within which it occurs. Inspection of the results in Table I shows that such is not the case. To wit, replacement of an acetyl group in position P_3 by an acetylprolyl group in position P_4 produces a free energy change of −1.20 ± 0.1 kcal/mol in the Phe–Phe series (U-73777E to U-71909E), whereas the same substitution in the Leu-Val alcohol series produces a free energy change of −3.2 ± 0.3 kcal/mol (U-80631E to U-77647E) and a free energy change of 0.4 ± 0.2 kcal/mol in the statine series (U-72407E to U-72408E).

If the affinity constant cannot be factored out into independent, intrinsic, individual components, then the question arises as to the forces which determine the affinity as measured by the K_d's. Inspection of Table I shows that $\Delta G°$ has, in general, a comparatively small value, resulting from the difference between large $\Delta H°$ and $T\Delta S°$ values. Thus, minor changes in $\Delta H°$ and $\Delta S°$ lead to important changes in $\Delta G°$. For instance, for a given $\Delta H°$ of about 14 cal/mol the $\Delta G°$ can be as little as 8.6 kcal/mol and as large as 10 kcal/mol (U-80215E and U-72408E, respectively). Conversely, the same $\Delta G°$ can be observed for widely different $\Delta S°$ and $\Delta H°$ values (e.g., U-77647E and U-71909E). These considerations are dramatically illustrated in Figure 4, which shows that there is no relationship between $\Delta H°$ and $\Delta G°$.

At first, one would not expect any correlation between the $\Delta H°$ and $\Delta S°$ values, since the basic forces responsible for the interaction of each amino acid with its binding site can be of different natures, each with arbitrary $\Delta H°$ and $\Delta S°$ values. However, if one finds a linear correlation, it then implies that the interaction of all amino acid side chains used in this study must be of the same basic nature. In other words, for each interaction, the value of the Δh determines the value of Δs. The large positive $\Delta H°$ and

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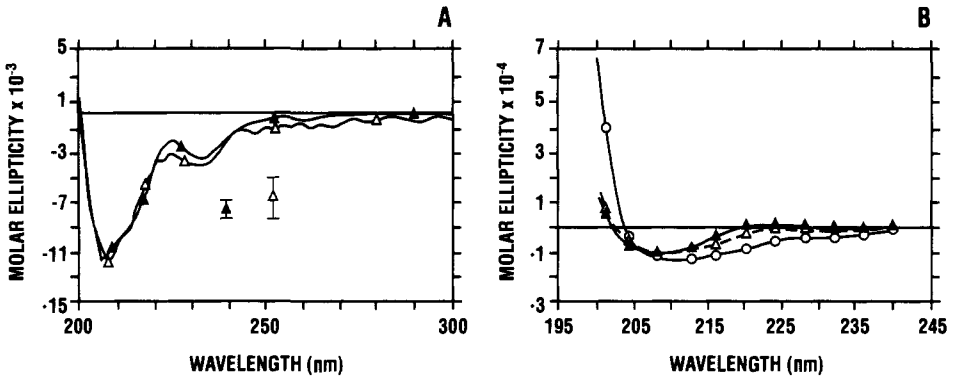

Figure 6. Circular dichroism spectra of U-71909E and U-77451E. CD spectra were recorded and analyzed by using the procedures described in the Experimental Section: panel A, (△) U-71909E and (▲) U-77451E; panel B, the temperature dependence of the molar ellipticity of U-71909E was studied as a function of temperature: △, 4 °C; ▲, 25 °C; ○, 75 °C.

$\Delta S°$ for binding (negative for dissociation), i.e., the increase in affinity with increasing temperature, indicate that for all renin inhibitors studied and for all side chains used the mode of binding is primarily hydrophobic in nature. However, an important characteristic of hydrophobic binding seems to be missing, namely the temperature dependence of $\Delta H°$, normally associated with this mode of binding.[14] At the present time we have no explanation for this behavior. Therefore, there must be additional, other than hydrophobic, interactions present. The calculated free energies of binding reflect differences between large enthalpy and entropy values and as such are sensitive to small deviations in the system. This sensitivity would not be predicted by the independent subsite model. The approximately linear dependence of $\Delta H°$ vs $\Delta S°$ is, in fact, a common occurrence in small molecule–protein interactions (see refs 15 and 16). A variety of causes can be ascribed to such a relationship, but all of them appear to be linked to the interaction of water with the peptide in solution.[15] In fact, it has been proposed that "the linear enthalpy–entropy relationship be used as a diagnostic test for the participation of water in protein processes".[16]

A priori, there can be several reasons why, in the case of renin, the $\Delta G°$'s fail to reflect the characteristics of the ligand–subsite interactions alone. Several additional phenomena might contribute to the value of the $\Delta G°$: (1) cooperative conformational changes at the active site upon binding of the ligand, (2) deviations from pure competitive behavior, (3) contribution of ligand–enzyme complexes other than the one in which the amino acid moieties are aligned with the optimal binding sites (i.e., binding "out-of-register"), and (4) cooperative intrapeptide interactions within the ligand moieties in solution and/or in the enzyme–inhibitor complex. Drastic conformational rearrangements probably do not occur since the information available concerning enzyme–ligand interactions at the active site of aspartyl proteases, in general, indicates that the inhibitors bind in the extended β-conformation. In that conformation, they are located in a position in which they could undergo only limited interactions.[19-23] In fact, the hexapeptide inhibitor, when bound to the HIV-1 protease, does not show any side chain–side chain interaction.[23] Even if the renin binds a certain class of inhibitors in such a way that side chain–side chain interactions could occur, it is not likely that the particular interaction would dominate these binding energies of the peptide. On the basis of the same information, one could rule out important contributions from the "out-of-register" binding. Experimental evidence rules out the noncompetitive or nonclassical binding modes. Therefore option 4 is the most likely candidate to explain our data.

In thermodynamic terms, two energy barriers must be overcome in order for an inhibitor to bind to an enzyme: (1) the free energy required for the removal of water from the enzyme active site such that the inhibitor can bind, and (2) the free energy required for the removal of solvated water from the regions of the inhibitor which will interact with the enzyme, together with the free energy changes associated with the conformational change going from the solution conformation to the bound conformation. If the solution conformation is different by a large amount of free energy from that of the bound form, then binding would be made unfavorable by this difference. Alternatively, the $\Delta G°$ of the solution conformation does not have to be different from that of the bound form. Binding will also be unfavorable when multiple solution conformations exist that are equal to the $\Delta G°$ of the bound conformation, if only a limited number can bind. A theoretical treatment of this alternative has recently been published.[24] On the other hand, if the solution conformation closely approximates the active site bound conformation, the binding of the inhibitor to the active site would be more thermodynamically favored.

We suggested above that, in the case of small linear peptide inhibitors of human renin, our thermodynamic binding data are best explained by assuming conformational differences in the peptides in solution (option 4

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above). The CD results (Figure 3, parts A and B) indicate that two structurally similar inhibitors (U-71909E and U-77451E) do not exist as random coils in solution but rather have characteristics of folded structures in solution. Temperature-dependent CD experiments confirmed the room temperature results, that is, that both peptides existed as something other than random coils in solution, since the CD intensities were temperature-dependent. It is not possible to say, with certainty, what type(s) of folded structure these peptides formed in aqueous solution; rather, we are concerned only by the fact that they *do not* exist as random structure since the compounds do exhibit some CD structure.

Previously, small linear peptides were thought to exist primarily as random coils in solution. Recent NMR work[17] has changed this perception. In addition, further evidence for the existence of renin inhibitor peptides as folded structures was recently presented.[18] Using cryogenic CD and NMR techniques, the authors were able to demonstrate a two-state conformation equilibrium (two predominant conformations) of a linear inhibitor of human renin in solution. NMR experiments were able to assign the region of conformational change as cis–trans isomerization about a Pro amide bond. Since 10 of 13 of our renin inhibitors contain either single or multiple proline residues, it is likely that a similar situation is occurring in our hands.

We are not questioning the existence of subsites in the active site of human renin, but the present data constitute a serious warning concerning the use of K_d's in structural variation studies regarding the shape of binding sites. The presence of a large extended binding site would, necessarily, make it more difficult to reach valid conclusions from K_d data alone. The original subsite model, as proposed by Schechter and Berger for papain, was based on a six-residue binding site, whereas human renin has an eight-residue extended binding site (see Figure 2). The subsite model has two major implications: (1) $\Delta H°$ and $\Delta S°$ should correlate with $\Delta G°$, and (2) a single change in specific position of a peptide inhibitor sequence should always have the same relative effects on the thermodynamic binding parameters. Our results show that neigher implication is valid for human renin, perhaps because of the solution conformations of the peptides. For other types of enzymes, such as angiotension converting enzyme, the interaction between side chains in the enzyme–inhibitor complex might also contribute to deviations from additivity. Although we have pointed out some deficiencies in the subsite model, our comments should not be construed as being entirely negative. On the contrary, they point to an important factor in the design of peptide inhibitors to human renin and possibly to other aspartyl proteases. The goal should be able to design a compound whose conformation in solution closely approximates the active, bound conformation. The rational design of inhibitors, therefore, should be based on optimizing the solution conformation as well as the interaction of the peptide with the active site. We feel that the deviations from the linear $\Delta H°$–$\Delta S°$ correlation should identify peptides for which the solution conformation approximates that of the peptide bound to the active site. Such compounds would be represented by points situated below the linear-regression curve given in Figure 5. Once peptides with such favorable conformations are found, the determination of their solution conformation by physical methods should provide the model on which conformationally constrained peptides with ideal inhibitory properties could be designed.