Structural basis of neurophysin hormone specificity: Geometry, polarity, and polarizability in aromatic ring interactions

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Abstract

The structural origins of the specificity of the neurophysin hormone-binding site for an aromatic residue in peptide position 2 were explored by analyzing the binding of a series of peptides in the context of the crystal structure of liganded neurophysin. A new modeling method for describing the van der Waals surface of binding sites assisted in the analysis. Particular attention was paid to the unusually large (5 kcal/mol) difference in binding free energy between Phe and Leu in position 2, a value representing more than three times the maximum expected based on hydrophobicity alone, and additionally remarkable since modeling indicated that the Leu side chain was readily accommodated by the binding pocket. Although evidence was obtained of a weak thermodynamic linkage between the binding interactions of the residue 2 side chain and of the peptide α -amino group, two factors are considered central. (1) The bound Leu side chain can establish only one-third of the van der Waals contacts available to a Phe side chain. (2) The bound Phe side chain appears to be additionally stabilized relative to Leu by more favorable dipole and induced dipole interactions with nonaromatic polar and sulfur ligands in the binding pocket, as evidenced by examination of its interactions in the pocket, analysis of the detailed energetics of transfer of Phe and Leu side chains from water to other phases, and comparison with thermodynamic and structural data for the binding of residue 1 side chains in this system. While such polar interactions of aromatic rings have been previously observed, the present results suggest their potential for significant thermodynamic contributions to protein structure and ligand recognition.

Keywords: binding site modeling; hormone binding site; neurophysin specificity; phenyl ring binding

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Abbreviations: NP, neurophysin; BNP, bovine neurophysin; CD, circular dichroism; NMR, nuclear magnetic resonance; TLC, thin layer chromatography; HC, HyperChem; Cha, β -cyclohexylalanine; Tha, β -thienylalanine; Abu, α -aminobutyrate; Phe-TyrNH₂, L-phenylalanyl-L-tyrosine amide; Phe-PheNH₂, L-phenylalanyl-L-phenylalanine amide; Phe-HisNH₂, L-phenylalanyl-L-histidine amide; Phe-TrpNH₂, L-phenylalanyl-L-tryptophan amide; Phe-AlaNH₂, L-phenylalanyl-L-alanine amide; Phe-ValNH₂, L-phenylalanyl-L-valine amide; Phe-AbuNH₂, L-phenylalanyl-L- α -aminobutyrate amide; Phe-ChaNH₂, L-phenylalanyl-L- β -cyclohexylalanine amide; Phe-MetNH₂, L-phenylalanyl-L-methionine amide; Phe-LeuNH₂, L-phenylalanyl-L-leucine amide; Phe-ThaNH₂, L-phenylalanyl-L- β -thienylalanine amide; Phe-p-OCH₃PheNH₂, L-phenylalanyl-L-(p-methoxy)-L-phenylalanine amide; Phe-p-I-PheNH₂, L-phenylalanyl-L-(p-iodo)-L-phenylalanine amide; Phe-3'I-TyrNH₂, L-phenylalanyl-L-(3'-iodo)-L-tyrosine amide; Phe-3'NO₂-TyrNH₂, L-phenylalanyl-L-(3'-nitro)-L-tyrosine amide; Phe-3',5'I-TyrNH₂, L-phenylalanyl-L-(3,5'-diiodo)-L-tyrosine amide; Phe-2'Cl-PheNH₂, L-phenylalanyl-L-(2'-chloro)-L-phenylalanine amide; Leu-TyrNH₂, L-leucyl-L-tyrosine amide; Leu-ChaNH₂, L-leucyl-L- β -cyclohexylalanine amide; Leu-LeuNH₂, L-leucyl-L-leucine amide; Leu-TrpNH₂, L-leucyl-L-tryptophan amide; p-iodo-Phe-TyrNH₂, L-(p-iodo)-L-phenylalanyl-L-tyrosine amide; Mes, (2-[N-morpholino]ethanesulfonic acid); EDAC, 1-ethyl-3(3-dimethylaminopropyl)carbodiimide.

The noncovalent interactions of the neurophysins (NPs) with the hormones oxytocin and vasopressin within their common precursors, or within processed neurophysin-hormone complexes, play an important role in NP folding and self-association, and consequently in the targeting and storage of the hormones within neurosecretory granules (e.g., Chaiken et al., 1983; Breslow & Burman, 1990). These interactions have been studied using the hormones, and also by using small peptides that have been shown by both solution studies (e.g., Breslow et al., 1973; Nicolas et al., 1978) and crystallographic studies (Chen et al., 1991; Rose et al., 1996) to mimic the hormones in their binding to NP and in their effects on NP conformation and self-association. A central feature of the interaction is its dependence on the presence of an aromatic residue in position 2 of ligand peptides. Tyr or Phe in this position confer essentially equivalent binding affinities, but no peptide containing a nonaromatic residue in position 2 has, until the present studies, been demonstrated to bind with measurable affinity (Breslow & Burman, 1990). The question, therefore, arises as to the structural basis of this specificity, particularly the extent to which it reflects steric as opposed to electronic properties of the binding site. For example, van der Waals contacts to the bound phenyl ring include those from

backbone carbonyl and -NH groups, a cystine sulfur and the carboxamide of Asn48, and binding-induced NMR shifts of the ring have suggested that these might perturb ring electronic structure (Breslow et al., 1995). Specific interactions between residue 2 ring electrons and NP might therefore contribute to stability.

The question also arises as to the exact thermodynamic contribution of the residue 2 ring to binding. The thermodynamic contribution of different segments of ligand peptides to binding has been determined by subtractive comparison of the binding energies of systematically related peptides, using the assumption—which has so far appeared valid, but which has been put to only limited tests—that the free energies of their individual contributions are additive (Breslow & Burman, 1990). However, since peptides containing Gly or Ala in position 2 do not bind with detectable affinities, only a minimum estimate of the thermodynamic contribution of the residue 2 aromatic ring has been obtained (Breslow & Burman, 1990). A similar situation prevails for the critical α -amino group of the peptide, which binds to NP via a salt bridge to Glu47 and three hydrogen bonds (Chen et al., 1991; Rose et al., 1996). These minimum estimates, when added to the more precise estimates of the contributions of other peptide residues, sum to an energy that is more than 12 kcal/mol more negative than the observed ΔG° of binding (Breslow & Burman, 1990). Potential contributions to this energy gap have been discussed (Breslow & Burman, 1990). Nonetheless, a better estimate of the thermodynamic contribution of residue 2 is needed, as is more information as to whether this contribution is additive with that of the other residues. The latter issue is particularly relevant in the light of the crystal structures of NP complexes. These indicate van der Waals contacts between the bound residue 2 tyrosine ring and side-chain carbon atoms of Glu47, the same residue that interacts with the peptide α -amino group.

In the present study, we have explored these issues by probing the specificity of the NP binding pocket using dipeptide amides containing different substituents at residue 2. Because the presence of an aromatic residue at position 1 increases the stability of NP-dipeptide complexes (Breslow et al., 1973), most dipeptides contained Phe at position 1. These studies differ from many of those earlier, in part by pushing the detection of binding to the limits of peptide solubility, and for the first time allow the detection of weak binding in the absence of an aromatic residue in position 2. Additionally, a new method for determining the van der Waals surface of a binding pocket is described and applied to the analysis of NP specificity. This method is a direct extension of the method of Lee and Richards (1971) for measuring solvent accessibility.

Finally, the specific contributions of Asn48 to the properties of the residue 2 binding pocket are investigated by comparison of the binding properties of sheep vasopressin-associated NP with those of its bovine analog. Bovine NPs have been the principal neurophysins investigated to date and, like almost all other NPs, contain Asn at position 48 (Chauvet et al., 1983). Sheep vasopressin-associated NP is almost identical to its bovine analog, but is singular in containing Ile at position 48 (Chauvet et al., 1983). (A minor substitution of Ile for the mixed Ile/Val combination at position 89 of the bovine protein is also present.) The sheep protein therefore represents a site-specific mutation of the polar Asn at position 48 to Ile.

Results

Peptide-binding studies

Table 1 reports the results of peptide-binding studies. Two considerations are initially relevant. First, the possibility exists that undetected impurities might bias the data. The binding affinity of

Table 1. Binding of peptides to mononitrated bovine neurophysins at pH 6.2, 25 °C^a

Peptide	Neurophysin	Observed binding constant (M ⁻¹)	−ΔG° (observed) (kcal/mol)
Reference peptides			
Phe-PheNH ₂	BNP-II	1.7 × 10 ⁴	6.0
Phe-TyrNH ₂	BNP-II	2.0 × 10 ⁴	6.1
Phe-TyrNH ₂	BNP-II ^b	1.1 × 10 ⁴	5.7
Leu-PheNH ₂	BNP-II ^b	1.2 × 10 ³	4.3
Phe-PheNH ₂	BNP-I	1.4 × 10 ⁴	5.8
Peptides with aliphatic residues in position 2			
Phe-AlaNH ₂	BNP-I ^b	NB (≤7.0)	(<1.2)
Phe-ValNH ₂	BNP-I ^c	7.6	1.2
Phe-ValNH ₂	BNP-II ^c	6.1	1.1
Phe-AbuNH ₂	BNP-II ^c	5.4	1.0
Phe-ChaNH ₂	BNP-II	NB (≤20)	(<1.8)
Leu-ChaNH ₂	BNP-II	NB (≤20)	(<1.8)
Phe-MetNH ₂	BNP-II	NB (≤22)	(<1.9)
Phe-LeuNH ₂	BNP-II ^{b,c}	2 ^d	0.4
Leu-LeuNH ₂	BNP-II ^c	NB (≤1)	(≤0)
Peptides with new or modified aromatic residues in position 2			
Phe-ThaNH ₂	BNP-II ^c	2.7 × 10 ³	4.8
Phe-p-OCH ₃ PheNH ₂	BNP-II ^c	30	2.1
Phe-p-I-PheNH ₂	BNP-II ^c	28 ^e	2.0
Phe-3'I-TyrNH ₂	BNP-II	970	4.2
Phe-3'NO ₂ -TyrNH ₂	BNP-II ^f	830	4.1
Phe-3,5'I-TyrNH ₂	BNP-II ^c	35 ^{e,g}	2.2
Phe-2'Cl-PheNH ₂	BNP-II	3.2 × 10 ³	4.9
Phe-HisNH ₂	BNP-II ^c	7.5	1.2
Leu-TrpNH ₂ ^h	BNP-II	90	2.8

^aStudies were carried out at a protein concentration of 0.2 mM in 0.1 M ammonium acetate buffer containing 2 mM Mes unless otherwise indicated. Peptides for which no evidence of binding was obtained are indicated as NB (nonbinding). Maximum binding constants for these peptides are given, calculated by assuming 10% protein saturation at the highest peptide concentration tested. Reference peptide results are those reported previously (Breslow et al., 1973, 1991). Data for Phe-AlaNH₂ were also reported earlier (Breslow, 1984).

^bStudies were conducted in 0.1 M KCl containing 0.05 M citrate or (for Leu-TyrNH₂ only) in 0.16 M KCl. Binding constants for BNP-II are ~40% lower in the presence of chloride ion and are the same in these two solvent systems; binding constants for BNP-I appear to be unaffected by chloride ion (Breslow et al., 1991).

^cBinding studies were conducted at a protein concentration of 0.1 mM or (for Leu-LeuNH₂ only) at a concentration of 0.05 mM. Binding constants obtained at 0.1 mM are expected to be 15 and 20% lower for BNP-I and -II, respectively, than those obtained at 0.2 mM concentration, as calculated from the dimerization constants of the two proteins in the unliganded state, assuming that the dimer binds peptide with 10-fold the affinity of the monomer as described elsewhere (Breslow et al., 1991). At 0.05 mM, BNP-II binding constants are 38% lower than at 0.2 mM.

^dDirect evidence of binding was also obtained by fluorescence (see Materials and methods), giving results similar to those obtained by CD.

^eThe low solubility of these peptides prevented fractional saturation of the protein above 4%. Evidence of binding was obtained, but the derived binding constant is approximate.

^fDetermined by gel filtration using the method of Hummel and Dreyer (1962) to avoid interference from the peptide nitrotyrosine in CD measurements. Non-nitrated protein at an initial concentration of 1 mM was used.

^gBinding studies were conducted at pH 5 to ensure complete protonation of diiodotyrosine. For bovine NP-II, binding constants at pH 5 are normally only 10% lower than at pH 6 (Breslow & Gargiulo, 1977).

^hResults reported are those obtained with peptide synthesized for this study (see Materials and methods) and are essentially identical to preliminary results reported earlier with a commercial sample (Breslow et al., 1973).

nitrated bovine NP-II for the peptide Phe-PheNH₂ is $\sim 2 \times 10^4$ M⁻¹ at pH 6 (Table 1). For nonbinding peptides containing Phe in position 1, the presence of less than 1% contamination with Phe-PheNH₂, or a related peptide containing Phe in position 2, would mislead; e.g., 0.15% contamination of 0.1 M nonbinding peptide with Phe-PheNH₂ would half saturate 2×10^{-4} M protein, generating an apparent binding constant of 10 M⁻¹. HPLC purification might not preclude such low levels of contamination. Accordingly, the binding constants of very weakly binding peptides should be viewed as maximum binding constants, except in the case of Phe-ValNH₂ and Phe-AbuNH₂, where controls rigorously ruled out the influence of impurities and/or the pH dependence of binding was inconsistent with expected impurities (see below). (The same consideration applies to the binding of Phe-LeuNH₂, the extremely weak binding of which made controls unreliable, this uncertainty in no way diminishing the arguments below.) Also relevant is that peptides that show no detectable binding to NP at maximum accessible concentrations nonetheless provide information about their maximum possible binding constants. These are calculated using the conservative assumption that 10% saturation with peptide might go undetected and thereby would represent the true binding at the highest peptide concentration used. (Earlier analyses (Breslow et al., 1980) had used a 20% value, which experience has shown is unrealistic.)

Table 1 indicates that only peptides with aromatic residues in position 2 bind with affinities >10 M⁻¹ under the conditions used. Peptides with Phe and Tyr in position 2 bind with the highest affinities, their binding constants similar as shown earlier (e.g., Breslow et al., 1991), despite the known hydrogen bonding (Chen et al., 1991; Rose et al., 1996) of the Tyr-OH group. The aromatic Thr residue, similar in size to Phe, binds with an affinity $\sim 1/9$ that of Phe or Tyr. Replacement of Tyr by the larger Trp in position 2 reduces binding affinity by a factor of 13 (compare Leu-TyrNH₂ and Leu-TrpNH₂). Replacement by His reduces binding by a factor of over 2,000, as suggested also by earlier results with a different His peptide (Breslow et al., 1973), reflecting at least in part the high hydrophilicity of the imidazole ring (see Discussion).

Substitution on the aromatic ring decreases binding affinity (Table 1). The 4' (para) position is the most sensitive to substitution, substitution at this position by either -I or -OCH₃ reducing binding by a factor of ~ 500 . Nitration or iodination of the 3' or 5' position each reduce binding by a factor of ~ 20 , while di-iodination at these positions leads to an ~ 500 -fold decrease in affinity. Substitution of Cl at the 2' position is better tolerated, leading to only a sixfold decrease in affinity. These effects will be seen below to parallel pocket shape.

When residue 2 was substituted by a nonaromatic residue, no binding was detected with either Cha or Met at accessible concentrations (Table 1). Earlier studies (Breslow, 1984; Breslow & Burman, 1990) had found no detectable binding by either Phe-AlaNH₂ (see also Table 1) or 2-Ala-oxytocin. Similarly, no binding was found earlier by Leu-GlyNH₂ (Breslow et al., 1973), 2-Gly-oxytocin, or 2-Ile-oxytocin (Breslow & Abrash, 1966), although the hormone analogs were tested only over a limited range of concentration. In the present work, Phe-ValNH₂ and Phe-AbuNH₂ (binding constants = 5–8 M⁻¹) were the first peptides lacking an aromatic residue in position 2 found capable of binding, with subsequent studies providing evidence of binding Phe-LeuNH₂ with even weaker affinity (apparent binding constant = 2 M⁻¹). In Table 2, the results of binding studies with aliphatic residues in position 2 are summarized as the loss in the negative free energy

Table 2. Thermodynamic effects of substituting Phe in peptide position 2 by aliphatic residues^a

Residue 2	Binding observed?	$\Delta\Delta G$ (kcal/mol)
Phe	+	0
Val	+	4.5–4.8 ^b
Abu	+	4.8
Leu	+	5.1 ^c
β -Cha	–	>4.2
Met	–	>4.1
Ala	–	>4.6

^aResults are expressed as the loss in binding free energy ($\Delta\Delta G$) resulting from the substitution. Data were obtained from results in Table 1 by comparison of the binding of Phe-PheNH₂ with Phe-XNH₂, but are corrected for differences in binding constants arising from differences in protein concentration or buffer as described in footnotes to Table 1.

^bThe reported range reflects small calculated differences between bovine NP-I and -II.

^cCorrections for the presence of buffer chloride ion (0.1 M) in these studies (Table 1) do not include the fact that the peptide itself was the chloride salt and was present at concentrations ranging from 0.025 to 0.1 M concentration. The lack of an additional correction is based on the fact that no significant differences in binding to bovine NP-II are found in buffers containing 0.1 M and 0.16 M KCl (Table 1).

of binding associated with the change from Phe to each aliphatic residue.

Concerns about potential contaminants in highly purified peptide preparations (vide supra) led us to test the apparent binding of purified Phe-ValNH₂ by several routes. First, a commercial custom preparation of peptide, synthesized by solid phase methodology and supplied to us with a chromatographic purity of $>99\%$, gave the same binding constant as the peptide we synthesized by solution methods. The binding constant was unchanged following additional HPLC purification. Second, high concentrations of non-nitrated NP failed to reduce the ligand available for binding by nitrated NP in the peptide preparations. Binding studies in Table 1 were conducted using 0.1–0.2 mM nitrated NP and 4–80 mM Phe-ValNH₂. If the binding observed in the presence of Phe-ValNH₂ were due to traces of a strongly binding contaminant as described above, addition of 1 mM non-nitrated NP to the binding reaction would reduce the free ligand concentration by 80–90% and similarly reduce the observed binding by the nitrated protein. Such reduction would not occur if binding were due to Phe-ValNH₂ with a binding constant of 6–8 M⁻¹. This experiment was carried out with both the purified peptide prepared by solution methods and that prepared by solid phase methods. In neither case was competition observed.

The binding of Phe-ValNH₂ was also probed by NMR. Proton spectra (not shown) of 1 mM Phe-ValNH₂ alone and in the presence of 0.05 mM bovine NP-I indicated an average broadening of 1.7 Hz for the Val methyl signals in the presence of NP at pH 6; broadening was reduced to 0.2 Hz at pH 2, qualitatively consistent with the expected decrease in binding at the lower pH (Breslow & Gargiulo, 1977). The results are incompatible with attribution of binding to a trace contaminant. Similar studies with Phe-LeuNH₂ found no unambiguous broadening of its methyl proton signals, in accord with its weaker binding. Together, the results provide the first evidence that peptides lacking an aromatic residue in position 2 can bind to the hormone binding site.

Nature of the complexes formed with Phe-ValNH₂ and Phe-AbuNH₂; evidence of a linkage between binding of residue 2 and the α -amino group

Ellipticity changes in Tyr49 on binding Phe-ValNH₂, Phe-AbuNH₂, and Phe-LeuNH₂ were consistent with the view that these peptides bind in the normal mode, but the Val/Abu peptides differed subtly from those with aromatic residues in position 2 in the pH dependence of binding. (Binding of the Leu peptide was too weak for pH studies.) The binding constant for peptide-NP interaction decreases above pH 6, reflecting the requirement for a protonated α -amino group in the interaction (e.g., Camier et al., 1973). The Val/Abu peptides appeared to behave similarly, although only a limited pH range could be examined due to decreased peptide solubility at high pH. Below pH 6, the binding of peptides with Phe or Tyr in position 2 again decreases, in this case due to protonation of the active site carboxyl group; protein containing a protonated carboxyl remains capable of binding, but with a markedly reduced affinity (Breslow & Gargiulo, 1977; Blumenstein et al., 1979). In the present studies, the binding constants of peptides containing Val or Abu in position 2 also decreased on lowering the pH from 6 to 3, but significantly less so than those of peptides with Tyr or Phe in position 2 (Table 3). This difference in pH dependence is another indication that the apparent binding of the aliphatic residues is not due to contaminants with Phe in position 2, and also indicates a subtle linkage between binding of the α -amino group and of residue 2.

Table 3 also demonstrates subtle differences in pH-dependence between peptides containing Phe and Tyr in position 2, most readily apparent by inversion of the relative affinities of Phe and Tyr peptides between pH 6 and pH 3. The differences in pH dependence among the peptides reflect differences in relative peptide affinity for the protonated and deprotonated states of Glu47 (Table 3) and qualitatively parallel differences among the peptides in their potential interactions with the pocket at neutral pH. Phe cannot form the hydrogen bond in which the Tyr-OH is involved; the Val and Abu peptides are too short to interact with Glu47 analogously to Tyr. Since binding to the protonated state involves rearrangement of the complex relative to its neutral pH conformation (Balaram et al., 1973; Live et al., 1987), the most direct explanation of these differences is that they represent differences

in salt bridge stability in the neutral pH complex that are not paralleled by differences in the low pH complex. In the context of this explanation, the fact that the preference of the Val and Abu peptides for binding to protonated relative to deprotonated species of Glu47 differs by a factor of three from that of Phe peptides (Table 3) indicates a free energy decrease of ~ 0.7 kcal/mol in the stability of the neutral pH salt bridge in the Val/Abu complexes. The Tyr-OH, which restricts rotation of the phenyl ring in the binding pocket (Blumenstein et al., 1980; 1981), increases the apparent salt bridge stability by ~ 0.5 kcal/mol.

Modeling of the binding pocket for the residue 2 aromatic ring

The decreased binding affinity associated with substitution on to the residue 2 aromatic ring, or substitution by an aliphatic residue, suggest a tight binding pocket, with significant energy barriers to expansion. To further analyze specificity, a new method was developed for delineating the van der Waals surface of the binding pocket. The method utilizes a probe of 1.2 Å radius to map the contours of the binding pocket, in the present case using the coordinates of subunit A from the crystal structure of the dipeptide complex of bovine NP-II (Chen et al., 1991) as specified in Materials and methods.

The geometry of the binding pocket for the Tyr ring of Phe-TyrNH₂ is best seen in the Kinemage (see Supplementary material in electronic appendix). Briefly, however, Figure 1 shows the binding cavity surface contours of five slices through the pocket; the dots represent the centers of the bound phenyl ring carbon atoms at each of these positions, and distances from the dots to the surface represent distances from the ring carbon centers to the van der Waals surface of the cavity. The Tyr-OH group is not shown since it is not accessible to the probe. Distances of the carbon

Table 3. Effect of pH on the binding of peptides to 0.1 mM mononitrated bovine NP-II at 25 °C^a

Peptide	Binding constant (M ⁻¹)		Binding constant ratios	
	pH 6.2	pH 3.0	pH 3/pH 6	COOH/COO ^b
Phe-TyrNH ₂ ^c	1.7×10^4	1.1×10^3	0.06	0.03
Phe-PheNH ₂ ^c	1.4×10^4	1.4×10^3	0.10	0.07
Phe-ValNH ₂	6.2	1.6	0.26	0.23
Phe-AbuNH ₂	5.4	1.3	0.24	0.21

^aConditions: 0.1 M ammonium acetate/2 mM Mes buffer, pH 6.2 or 0.1 M Na phosphate buffer, pH 3.

^bRelative affinity of peptide for protein containing protonated and deprotonated forms of Glu47. Results are calculated from the pH 3/pH 6 ratio, by subtracting from the ratio the expected contribution to binding of the ionized carboxyl at pH 3. This contribution (0.03) is calculated assuming a pK_a of 4.5 for Glu47 in unliganded bovine NP-II (Zheng et al., 1996).

^cData at pH 6.2 for these peptides were calculated from data obtained at 0.2 mM protein concentration as described in Table 1.

Fig. 1. Scaled representation of the surface of the binding cavity for the Tyr2 ring at different positions along the z-axis of the ring (representing the axis between CG and CZ and shown perpendicular to the plane of the page). The dots represent the centers of the ring carbons at the individual positions. The ruler represents a distance of 4 Å: (A) CZ; (B) CE; (C) CD; (D) CG.

atoms to the contour lines are in excellent agreement with the X-ray distances of the atoms involved. The most noticeable anomaly is in Figure 1C where the center of CD2 of the Tyr ring is only 1.2 Å from the contour to its right representing OD1 of Asn48. The close separation of these two atoms from the X-ray data (3.1 Å) might result from distortion of Asn48 in the crystal packing or hydrogen bond-like interactions with the ring (see Discussion). In either case this distance is not constant when different subunits are compared either in the dipeptide complex or in the oxytocin complex (e.g., Table 4). As the pocket progresses from the CE atoms of the ring to CD and CG, the width and height of the pocket increase, thus reducing the magnitude of the van der Waals interaction energies (essentially zero for CG). Between CG and CB, the contours of the pocket flare out, and the pocket makes contact with bulk solvent at about 80% along the way from CG to CB (not shown).

Figure 1 allows calculation of the dimensions of the binding pocket in the different slices. At the CZ position, the maximum dimensions along the *x* and *y* axes are 5.9 and 3.9 Å, respectively, which can be compared with the van der Waals dimensions of a phenyl group at this position (including hydrogens) of 5.8–5.9 Å and 3.5–3.7 Å, respectively. The fit of the ring at this position is therefore very tight along both axes. At CE, the calculated maximum dimensions are 6.8 and 4.0 Å along the *x* (width) and *y* (height) axes, respectively, although the actual pocket height in the center of the bound ring calculates to 3.5 Å. For comparison, the *x,y* dimensions of a phenyl group at this position are 6.4 and 3.5–3.7 Å, again indicating a very tight fit. At CD, however, the

width and maximum height of the pocket increase to 7.0 and 5.2 Å, respectively, indicating a particularly large increase in the space available to ligand in the *y* dimension. At the CG position, the pocket size has markedly increased in all dimensions (vide supra). A different view is provided by Figure 2, which describes the area accessible to the 1.2 Å radius probe for several slices in the absence and presence of the bound tyrosine ring. Note that the accessible area is less than the area encompassed by the cavity surface (e.g., Fig. 1) because of the van der Waals radius of the probe (see Materials and methods). In the unbound state, slices taken at positions corresponding to CZ, CE, and CD of Tyr clearly show the accessibility of the cavity to the probe; this is also true at CG (not shown). In the bound state, the cavity is almost completely filled by the phenyl ring; the CZ and CE ring atoms of Tyr are completely inaccessible to the probe (and therefore to solvent), while the CD1 position has very limited accessibility. The CG of Tyr (not shown) is inaccessible in the bound state, in large part because of shielding by other elements of Tyr.

Table 4. Van der Waals interactions of the bound residue 2 phenyl ring with noncarbon atoms of bovine NP-II^a

Phenyl ring position	Protein atom	Distance (Å)	
		A	B
CD1	O, Phe22	3.8	4.0/4.7
CD1	N, Gly23	3.9	3.8/4.1
CD1	S, Cys10	3.9	3.8/3.8
CD2	N, Pro24	3.9	4.0/3.9
CD2	OD1, Asn48	3.1	4.8/3.3
CD2	ND2, Asn48	3.7	3.5/4.9
CE1	N, Gly23	3.4	3.4/3.6
CE1	S, Cys10	3.7	3.8/3.7
CE1	O, Phe22	3.7	4.3/4.9
CE1	O, Cys21	3.6	4.3/4.6
CE1	O, Gly23	3.6	4.1/4.2
CE2	O, Gly23	3.9	4.4/4.2
CE2	N, Pro24	3.5	3.4/3.3
CE2	O, Cys44	3.3	4.9/3.8
CE2	OD1, Asn48	3.8	4.4/3.3
CE2	ND2, Asn48	3.8	3.7/4.5
CZ	O, Gly23	3.3	3.7/3.7
CZ	N, Pro24	3.6	3.6/3.6
CZ	O, Cys44	3.5	3.6/3.6

^aData (column A) are from subunit A of the complex with p-iodo-Phe-TyrNH₂ (see Materials and methods) and (column B) from the oxytocin complex (Rose et al., 1996). The two sets of data for the oxytocin complex represent data sets for the two different chains of the dimer. Only contacts representing distances ≤ 4 Å in either the dipeptide complex or in one or both chains of the oxytocin complex are included.

Fig. 2. Representation of the binding cavity area available to a 1.2 Å radius probe at selected positions along the *z*-axis of the Tyr2 ring in the absence and presence of Tyr2. Slices are labeled according to the ring atoms of Tyr2 at that position in the bound state. The neurophysin subunit is shown in white and the probe-accessible area is shown in black. The *z*-axis of the Tyr2 ring is perpendicular to the plane of the paper. At the CZ and CE positions, the cavity is probe-accessible only when Tyr2 is absent. At the CD position, a very small probe-accessible area remains at Tyr CD1 in the bound state. Left column from top to bottom: CZ, CE, and CD positions in the absence of ligand. Right column from top to bottom: same as left column, but in the presence of Tyr2 in the binding pocket.

Role of Asn48 in the binding pocket

To determine the potential contribution to binding of the contacts between Asn48 and the bound phenyl ring, comparison was made between bovine NP-II (Asn48) and sheep vasopressin-associated NP (Ile48). Table 5 indicates that substitution of Ile for Asn has largely minor effects on NP binding and dimerization properties, and on the binding-induced change in the NMR chemical shift of peptide phenyl ring CE protons. The most obvious differences between the two proteins were 50% and 150% increases by the sheep protein in the binding constants for Phe-PheNH₂ and Phe-ValNH₂, respectively. No binding of Phe-LeuNH₂ by the sheep protein was observed, but the limitations of the methodology used (Table 5) is such that binding affinities equal to or twice that of the bovine protein are not excluded.

Discussion

Solvent accessibility and steric hindrance in the specificity for residue 2

Both the inaccessibility to solvent of most of the bound residue 2 side chain and the potential for steric hindrance by the tight binding pocket contribute to binding specificity. Solvent inaccessibility finds its clearest manifestation in the weak binding of the aromatic but polar His side chain, a phenomenon discussed more quantitatively below. Steric hindrance contributes to the weaker binding both of substituted aromatic rings (see also below) and of aliphatic side chains. The virtual identity of the pocket height to the thickness of a phenyl group from the CE plane outward to CZ narrowly allows acceptance of nonpolar aromatic rings, but presents a steric barrier to intrinsically bulkier aliphatic side chains that would extend to the CE position; e.g., no measurable binding is seen for Met and Cha. Modeling of the substitution of a cyclohexane ring (with its associated H atoms) for a phenyl ring (with its H atoms) demonstrates the problem with the Cha peptide. Complete insertion of the cyclohexyl group into the pocket can be achieved without unfavorable van der Waals contacts, and with stabilizing contacts similar to those calculated for Tyr, but only at the expense of bond distortion. The calculated unfavorable 5.9 kcal/mol difference between bound and unbound conformations is >5 kcal/mol greater than the 0.75 kcal/mol strain associated with binding Tyr (see

Materials and methods). Analogous modeling results with Cha were also obtained with the two subunits of the oxytocin complex, although subunit G of the dipeptide complex (probably as a result of crystal packing distortion) appeared to allow insertion without significant strain.

In contrast to the Cha side chain, modeling indicates that the side chain of Leu, with its associated H atoms, is readily accommodated by the pocket. This reflects the fact that the Leu side chain does not extend fully into the phenyl ring CE position, but penetrates only slightly beyond CD, at which position the vertical dimension of the pocket is sufficient to accommodate the additional hydrogens and altered bond angles involved. Calculations indicate only 0.73 kcal/mol conformational strain for the bound Leu side chain (the same as for Tyr), with only a single trivially unfavorable contact to NP (0.028 kcal/mol), and with stabilizing van der Waals contacts as discussed below. Modeling of bound Val and Abu peptides was more complex. For the present, attention will primarily be directed to the Leu peptide.

Role of pocket shape in the quantitative discrimination between Phe and Leu

If there is no steric hindrance to the binding of Leu, why does it bind so weakly? The 5.1 kcal/mol weaker binding of Leu than of Phe (Table 2) represents a very large discrimination between these two residues, one that exceeds the binding specificity of some phenylalanyl aminoacyl tRNA synthetases (e.g., Freist et al., 1996). An appreciation of the surprisingly high value can be found by converting this value to cal/mol per Å² of the 30 Å² difference in accessible surface area of the two side chains measured in N-acetyl amino acid amides (Sharp et al., 1991). The value of 170 cal/mol/Å² can be compared with estimates of 20–50 cal/mol/Å² (e.g., Sharp et al., 1991; Eriksson et al., 1992) in different hydrophobic models.

A standard model for the binding of a nonpolar side chain into a protein pocket is its transfer from water to octanol (Fauchere & Pliska, 1983). However, the free energies of transfer of the side chains of Leu (−2.3 kcal/mol) and Phe (−2.4 kcal/mol) from water to octanol are essentially identical (Fauchere & Pliska, 1983; Eisenberg & McLachlan, 1986). Additionally, assuming similar conformations for unliganded Phe and Leu peptides, energetically uphill changes in translational and total rotational entropy associated with binding (e.g., Morton et al., 1995) can be shown not to

Table 5. Comparative properties of vasopressin-associated neurophysins from the cow and the sheep

	Cow	Sheep
Binding constant (M ^{−1}) for Phe-PheNH ₂ at pH 7.3 ^a	7.5 × 10 ³	1.1 × 10 ⁴
Binding constant (M ^{−1}) for Phe-ValNH ₂ at pH 6.2 ^b	4.4	11.0
Binding constant (M ^{−1}) for Phe-LeuNH ₂ at pH 6.2	2 (from Table 1)	≤5 ^a (no evidence of binding)
Binding-induced change in chemical shift of CE ring protons of peptide Tyr2 ^c	−0.56 ppm	−0.58 ppm
Dimerization constant, pH 6–7 ^d	5.8 × 10 ³	4.4 × 10 ³

^aDetermined by fluorescence (see Materials and methods) using 0.06 mM unmodified protein.

^bThe binding constant of the sheep protein was determined by CD using 0.04 mM nitrated sheep NP. The constant reported for bovine NP-II is that in Table 1 corrected to a protein concentration of 0.04 mM.

^cDetermined by NMR as described earlier (Breslow et al., 1995).

^dDetermined by NMR. The methodology used and the dimerization data for the bovine protein are those reported earlier (Zheng et al., 1996).

Fig. 3. Cartoon illustrating the relative positions of the side chains of Phe and Leu in the binding cavity. Energy values are the calculated van der Waals energies associated with contacts between the protein and the demarcated regions of the ligand, considering only contacts at ≤ 4 Å and treating aromatic and nonaromatic carbons similarly. The increase in cavity size on going from the CE position to the CD position of Phe is associated with a diminution in van der Waals contact energy at the CD position. The side chain of Leu extends into the pocket between the CD and CE positions and is associated with similar calculated packing energies as the CD position of the phenyl ring with this method of calculation.

contribute materially to the weaker binding of Leu. It is possible, but not established, that a small fraction of the difference between Phe and Leu reflects, as in the case of Val and Abu peptides, an effect on the stability of the salt bridge. Of the remainder, a significant contribution can be shown to arise from the effects of pocket geometry on the van der Waals interactions available to Phe and Leu side chains. Figure 3 is a two-dimensional cartoon of the binding pocket with Phe and Leu side chains. The CZ and CE positions of Phe insert into the tight end of the pocket, with the CD position at the wider end, while the terminal methyl groups of Leu penetrate to a region between CD and CE of Phe (*vide supra*). Analysis of the distribution of van der Waals interactions for the bound Phe ring (Fig. 3) indicates that two-thirds of the total ring van der Waals binding energy comes from contacts to the CZ and CE positions, and only one-third from the CD and CG positions. Moreover (Fig. 3), the van der Waals contacts of the bound Leu side chain are energetically similar to those of the CG and CD positions of Phe when no distinction is made between aliphatic and aromatic interactions. Thus, the packing interactions of a Leu side chain will be significantly less stabilizing than those of Phe based on pocket geometry alone.

Figure 3 also indicates that substitution of Leu for Phe will leave a cavity in the CZ,CE region of the binding pocket. This cavity is too small to contain water. Figure 4 depicts the space available in the pocket to a probe having the radius of a water molecule (1.4 Å) when the pocket is occupied by Leu. The lack of space for water

Fig. 4. Water-accessible area of NP when Leu is positioned in the binding pocket. The method of calculation and the depiction of results are the same as in Figure 2 except that the probe radius is 1.4 Å, the radius of water. Slices are labeled according to Tyr ring positions when Tyr is the ligand. Left column, from top to bottom: (1) Tyr CZ; (2) Tyr CE. Right column, from top to bottom: (1) halfway between Tyr CE and CD; (2) Tyr CD. The binding cavity (*cf.* Fig. 2) is totally inaccessible to water at all positions except Tyr CD, where a very small probe-accessible area is barely perceptible. (Note that the small cavity in the slice between CE and CD is not in the binding pocket.)

in the region corresponding to CZ,CE of Phe in part reflects the slight penetration of the CG,CD region of Leu into this region upon energy minimization. Most significantly, the absence of water in the cavity indicates that the energy change upon ligand binding associated with pocket dehydration per se is similar for Leu and for Phe, since the pocket is similarly dehydrated when Phe is bound (*e.g.*, Fig. 2). Thus, this energy change does not enter into the differences in binding of these two amino acids.

Role of Phe aromaticity in the discrimination between Phe and Leu

The above analysis omits consideration of thermodynamic differences between Phe and Leu that are masked by their similar free energies of transfer from water to octanol. The free energy of transfer of Phe and Leu side chains from water to octanol (Fig. 5) can be represented as the sum of two processes: (1) The side chain is dehydrated; *i.e.*, it is removed from water to the vapor phase, a process associated with the reorganization of water and the loss of van der Waals and other (see below) contacts between the side chain and water. (2) The side chain is transferred to octanol, a process associated with the reorganization of octanol and the establishment of contacts between the side chain and octanol. The sum of these two processes is energetically the same for the two side chains, and we assume that the solvent reorganization processes are also energetically similar for the two, since the two side chains occupy similar volumes (Sharp et al., 1991; Lee, 1993). However, the dehydration free energy of Phe is 3 kcal/mol more positive than that for Leu (Radzicka & Wolfenden, 1988), a dif-

Transfer from Water to Octanol (Kcal/mol)

PHE _{Water}	+ 0.8	PHE _{Gas}	- 3.2	PHE _{Octanol}	Total $\Delta G^0 = -2.4$
LEU _{Water}	-2.3	LEU _{Gas}	- 0	LEU _{Octanol}	Total $\Delta G^0 = -2.3$

Transfer from Water to Neurophysin (Kcal/mol)

PHE _{Water}	+ 0.8	PHE _{Gas}	- 7.4	PHE _{NP}	Total $\Delta G^0 = -6.6$
LEU _{Water}	-2.3	LEU _{Gas}	+0.8	LEU _{NP}	Total $\Delta G^0 = -1.5$

Fig. 5. Energetics of the transfer of Phe and Leu side chains between different phases, and their application to the energetics of binding to neurophysin at pH 6 and 25 °C. Free energies of transfer between water and octanol (Fauchere & Pliska, 1983; Eisenberg & McLachlan, 1986), between water and the gas phase (Radzicka & Wolfenden, 1988), and between water and neurophysin (this paper) are experimental values. Free energies of transfer between the gas phase and either octanol or NP are calculated from the other two sets of data. Note that the absolute free energies of binding the Phe and Leu side chains to NP are calculated as described in Discussion, but that only the difference between these two values is significant in this context.

ference that is enthalpically, not entropically, determined, with potential origins in hydrogen bonds between the protons of water and the aromatic ring (e.g., Ooi et al., 1987; Makhatazde & Privalov, 1994). Moreover, since the total free energy of transfer of Leu and Phe from water to octanol is the same, the difference in their hydration energies must extend to the differences in their interactions with octanol (Fig. 5); i.e., stabilizing interactions of the Phe side chain with octanol are 3 kcal/mol stronger than those of Leu. This extra stability of Phe interactions with octanol appears to reflect largely, but not completely, the polarity of octanol. In contrast to octanol, the free energy of transfer of a Phe side chain from water to cyclohexane is 2 kcal/mol more positive than that of a Leu side chain (Radzicka & Wolfenden, 1988), indicating that the 3 kcal/mol extra energy required to remove a Phe side chain from water is only partially compensated for by the increased strength (relative to Leu) of its interactions with cyclohexane. The difference between the octanol and cyclohexane data additionally argues that the difference between Phe and Leu in their interactions does not result solely from the ~50% difference (e.g., Sharp et al., 1991) in their side-chain accessible areas.

The difference in hydration energy between Phe and Leu enters into their relative binding free energies to NP (Fig. 5). The 5 kcal/mol difference in binding energy between Phe and Leu represents an 8 kcal/mol difference in the energetics of their interactions in the binding pocket, a difference that becomes more difficult to explain largely by numerical differences in van der Waals contacts. Thus, the van der Waals energies in Figure 3, calculated without distinction between aliphatic and aromatic carbons, suggest a difference in packing energy between Phe and Leu of 3.4 kcal/mol. Together with a potential 0.7 kcal/mol effect on salt bridge stability, this accounts for only half of the 8 kcal/mol. Moreover,

there are no protein aromatic rings in the binding pocket, precluding electronic interactions between aromatic rings, and contributions from ring H-bonding interactions appear small (see below). To address the discrepancy, we suggest that the fixed and induced dipole components of van der Waals contacts involving Phe aromatic carbons have energetically different consequences than the comparable contacts of Leu aliphatic carbons. In keeping with side-chain transfer free energies to octanol and cyclohexane, the contacts involved are proposed principally to represent those to more polar or polarizable groups, a number of which are shown below to be present in the residue 2 binding pocket. The interactions involved are also suggested to account for the fact that the binding of peptides containing aromatic residues in position 2 is always enthalpy-driven (Breslow & Gargiulo, 1977; Whittaker et al., 1985).

Analysis of the binding pocket in fact indicates that ~34% of the atoms and 40% of the close contacts between the phenyl ring and NP involve O, N, or S. Table 4 lists the closest interactions of the residue 2 phenyl group with atoms other than carbon in the dipeptide and oxytocin complexes. Since NMR indicates a similar Tyr2 ring environment in both complexes (Breslow et al., 1995), the invariant distances to the Cys10 sulfur and to backbone N atoms when the two complexes are compared suggests the particular importance of the sulfur and backbone N interactions. Note that with the possible exception of OD1 of Asn48 for which distances to the phenyl ring as short as 3.1 Å are measured, there is no indication of ring hydrogen bonding, and that contributions of the polar interactions of Asn48 to phenyl ring stabilization are minor. Substitution of Ile for Asn at position 48 in the sheep protein has only a trivial effect on the NMR behavior of the bound phenyl ring and diminishes the thermodynamic advantage for the binding of Phe over that of Val in position 2 by only 0.3 kcal/mol (calculated from Table 5). This apparent lack of importance is paralleled by differences between the two subunits of the oxytocin complex in distances to the carboxamide (Table 4), which can also be seen among the four subunits of the crystalline dipeptide complex (data not shown).

The potential role of polar interactions in stabilizing the residue 2 phenyl group binding finds support in the mode of binding of the phenyl ring of residue 1 of ligand peptides, coupled with thermodynamic data on the binding of this group. A Phe in position 1 confers a 1.4 kcal/mol binding free energy advantage relative to Leu and a 2.2 kcal/mol advantage relative to Ala. In both cases, the Phe advantage is enthalpy driven, while the advantage of Leu over Ala is entropy driven (Whittaker et al., 1985). Moreover, the crystal structure of the complex with p-iodo-Phe-TyrNH₂ (Chen et al., 1991) indicates that the Phe ring binds to a relatively open region of the protein with ~60% of the contacts between the ring and NP involving polar atoms (Table 6), the combined structural and thermodynamic data providing evidence of phenyl group interactions here that qualitatively and energetically differ from those of aliphatic side chains. Crystallographic evidence of aromatic ring interactions with oxygen and sulfur atoms (Thomas et al., 1982; Reid et al., 1985), with side-chain amide nitrogens (Burley & Petsko, 1988) and with cations (e.g., Dougherty, 1996) has been reported earlier. However, evidence for a thermodynamic contribution of interactions with neutral sulfur, oxygen, and nitrogen atoms is either absent or controversial (e.g., Dougherty, 1996). The Phe1 data represent experimental evidence of thermodynamic contributions from such phenyl ring interactions, the amide nitrogens in this case from the peptide backbone.

Table 6. Van der Waals interactions of the phenylalanyl ring of *p*-iodo-Phe-TyrNH₂ when bound to bovine NP-II^a

Ring position	Protein atom	Distance (Å)
CG	O, Ser52	4.1/4.1
CD2	O, Leu50	3.3/3.5
CD2	C, Pro51	3.6/3.7
CD2	O, Pro51	3.5/3.3
CD2	N, Ser52	4.0/4.3
CD2	C, Ser52	3.9/3.9
CD2	O, Ser52	3.8/3.6
CE2	C, Pro51	3.5/3.6
CE2	O, Pro51	3.0/2.8 (possible H-bond)
CE2	N, Ser52	4.0/4.4
CE2	C, Ser52	3.8/3.8
CE2	O, Ser52	4.2/4.0
CE2	N, Pro53	3.8/3.4
CE2	CD, Pro53	3.9/3.9
CZ	CD, Pro53	3.7/3.9

^aDistances listed are for subunits A and C of the re-refined crystal structure (see Materials and methods). Atoms listed without a following letter are backbone atoms.

Other aspects of specificity

Brief comments about several other aspects of the binding data are warranted. First, the 4.6 kcal/mol difference in binding energy between Phe and His, while qualitatively consistent with the greater hydrophilicity of His, is sufficiently high relative to predictions from solvent transfer free energies as to suggest that the different electron distribution in the Phe and His ring systems contribute to differences in their interaction in the binding pocket. Second, the binding of Trp, while perhaps surprising in view of its large ring size, is consistent with pocket dimensions. Modeling indicates that the Trp ring system can be accommodated with <1 kcal additional strain relative to Tyr by orienting it so that the five-membered ring is largely out of the pocket, while the six-membered ring is close to the position normally occupied by the Tyr phenyl group, with similar stabilizing interactions. Finally, while the effects on binding of substituents on the residue 2 aromatic ring generally parallel pocket dimensions, the fact that phenyl rings iodinated in the CE and CZ positions exhibit measurable binding is noteworthy. Iodination increases the thickness of the side chain by 0.6–0.7 Å, an amount that modeling suggests is not available in the native structure. The pocket therefore can expand or tolerate locally unfavorable interactions if sufficient stabilizing interactions are present, in this case provided by the phenyl ring and perhaps also by the highly polarizable iodine, but absent in the aliphatic residues studied to date.

Total energetic contribution of the residue 2 phenyl group

The present results allow an improved estimate of the free energy contribution of the residue 2 phenyl group to binding. The 5.1 kcal/mol difference between Phe and Leu is a lower limit, since contributions of the Leu methyl groups (and to a much lesser extent, the gamma position) must be subtracted from its binding. As judged by average observed methyl group contributions to protein stabil-

ity (Lee, 1993), the probable combined energetic contribution of the two Leu methyl groups can be estimated at 2.5 ± 1 kcal/mol. Using 1.5 kcal as a minimum, the total stabilizing contribution of the phenyl group is estimated as ≥ 6.6 kcal/mol. This value exceeds by over 2.5 kcal/mol the highest estimates we have seen (Sharp et al., 1991) of the hydrophobic contribution of a phenyl ring to protein stability. We assign a large part of this extra stability to polar interactions of the aromatic ring, the potential for which has been analyzed by Burley and Petsko (1988). The present system provides evidence of a thermodynamic contribution of such interactions to protein stability and ligand recognition.

Materials and methods

Protein preparation

Bovine NP-I and -II were purified as described elsewhere (e.g., Breslow et al., 1971; Sur et al., 1979). Sheep vasopressin-associated NP was prepared analogously to its bovine analog (BNP-II). Fractions from the Sephadex ion-exchange step that were contaminated with sheep oxytocin-associated NP (which differs from its bovine analog) were further purified by ion-exchange HPLC on a protein PAK DEAE-5PW column using a linear gradient beginning with A (0.02 M ammonium acetate, pH 7.4) and extending to 50% A, 50% B (0.5 M ammonium acetate, pH 4.6) at 35 min. The substitution of Ile for Asn at position 48 in the sheep protein was verified by its amino acid composition and by automated gas phase sequencing.

Peptide synthesis

Unless otherwise indicated, peptides were synthesized by coupling the carbodiimide-activated carboxyl group of the N_α-Boc derivative of residue 1 with the amino acid ester of residue 2, followed by conversion of the ester to the amide and deblocking. All steps were monitored by TLC. The synthesis of Phe-ChaNH₂ outlined here is representative. β-Cyclohexyl-L-alanine (1.5 g) was converted to its methyl ester by reaction with SOCl₂ in absolute methanol at 25 °C. After separation from residual reactants, the resulting ester was reacted with 0.83 mol equivalents each N,N-dicyclohexylcarbodiimide and N_α-Boc-L-phenylalanine in CH₂Cl₂. Following reaction for 22 h at 25 °C, the mixture was filtered, the filtrate washed with acid and weak base, the dried solvent evacuated and the residue dissolved in ethyl ether. The insoluble dicyclohexylurea was removed and the ether evacuated to give 2.12 g of the protected dipeptide ester. The ester was converted to the amide by reaction with NH₃ gas in methanol at 25 °C, the solvent removed and the protected dipeptide amide was deblocked in glacial acetic acid containing 1 M HCl. The vacuum-dried residue was mixed with ethyl ether and the white insoluble product separated by filtration and dried. The yield of dipeptide was 1.35 g. For binding studies, the product was purified on a C₁₈ column by HPLC with a gradient starting at 100% solvent A (0.1% TFA in water) and terminating at 40 min with 40% solvent A and 60% solvent B (0.1% TFA in CH₃CN), yielding (after lyophilization) the trifluoroacetate salt as the final product.

Analogous procedures were used to synthesize Phe-MetNH₂, Phe-ThaNH₂, Phe-3'I-TyrNH₂, Phe-2'Cl-TyrNH₂, Leu-ChaNH₂, Leu-TrpNH₂, Phe-p-I-PheNH₂, Phe-3',5'I-TyrNH₂, and Phe-p-OCH₃-PheNH₂, with the occasional substitution of EDAC as the coupling agent. For Phe3',5'I-TyrNH₂, reactions were shielded from light and base omitted from the coupling reaction to protect the

diiodo-tyrosine; no loss of iodine was detected in the final product. Phe-HisNH₂ was synthesized by conversion of the dipeptide methyl ester (Bachem, King of Prussia, Pennsylvania) to its Boc derivative using Boc-On (Aldrich, Milwaukee, Wisconsin), followed by conversion to the amide and deprotection as above.

Solution phase synthesis of Phe-ValNH₂ was achieved most efficiently by direct coupling of Boc-L-Phe with L-ValNH₂ (Bachem) using EDAC as above, followed by deprotection. Another lot of this dipeptide was custom synthesized by solid phase methodology and supplied in >99% purity by the manufacturer (Peptides International, Louisville, Kentucky). A fraction of this peptide was further purified by HPLC in this laboratory. Phe-AbuNH₂ was synthesized by solid phase methodology and supplied in >99% purity by Peptides International. These peptides were the trifluoroacetate salts.

Phe-3'-NO₂-TyrNH₂ and Phe-AlaNH₂ were obtained from Vega. Phe-PheNH₂ and Phe-TyrNH₂ were from Bachem. Phe-LeuNH₂ and Leu-LeuNH₂ were from Sigma. All were the hydrochlorides (see Results). Two different lots of Phe-LeuNH₂ were studied. Both gave the same binding constant by direct (see below) CD and fluorescence methods. One lot contained a fluorescent impurity and gave a higher apparent binding constant by competition (see below) than by direct methods. The second contained no such impurity and gave the same binding constant by both methods. The purity and identity of the second lot were confirmed by TLC, amino acid analysis, potentiometric titration (to confirm that its carboxyl group was in the amide form), and CD to verify chirality. The aberrant results with the first lot are attributed to the impurity.

Binding studies

Binding of peptides to the NP hormone-binding site was typically monitored by CD using purified mononitrated NP prepared as described earlier (e.g., Breslow et al., 1991). The method used (Breslow et al., 1973) is based on the large change in CD spectrum of the NP nitrotyrosine when the hormone-binding site is occupied. Because the magnitude of the change is related only to the degree of site occupancy, and is typically independent of peptide identity, and because the lowest energy nitrotyrosine signal occurs at wavelengths at which most peptides are transparent, the degree of binding site occupancy at any peptide concentration is directly determined from the CD spectrum relative to that of the unbound and fully bound states. In the case of weakly binding peptides, the CD spectrum of the fully bound state is determined by saturation with a strongly binding peptide. With the exception of desmopressin, which lacks an α -amino group and binds to NP in an aberrant manner because of its unusual structure (Wang et al., 1996), and which does not perturb the nitrotyrosine (E. Breslow & R. Deeb, unpubl. results), no differences among bound peptides in the nature of the perturbation of the nitrotyrosine signal have been detected (Breslow et al., 1973); this applies to the present study. Thus the ability of the method to detect weak binding constants is limited only by peptide solubility. Occasional direct binding studies were performed by monitoring the binding-induced change in Tyr49 fluorescence of the unmodified protein as described elsewhere (Sur et al., 1979). These studies were limited to peptides that did not contain tyrosine. For peptides that showed little or no detectable binding using direct CD and fluorescence methods, binding constants were also assessed by competition, measuring their effects on the binding constants of ligand peptides (e.g., Breslow et al., 1980).

NMR studies

One-dimensional proton NMR spectra were obtained at 300 MHz. Protein and peptide were individually dissolved in deuterated phosphate buffer, pH 6, lyophilized and redissolved in D₂O prior to mixing and spectral analysis. Spectra were also obtained at pH 2, following lowering of the pH with DCl. Dimerization of the sheep protein was also monitored by NMR, as described elsewhere (e.g., Zheng et al., 1996).

Binding pocket modeling

The coordinates used for modeling the binding pocket for residue 2 and the effects of substitutions are (unless otherwise noted) the original coordinates of subunit A of the tetrameric asymmetric unit of the crystalline complex of BNP-II with p-iodo-L-Phe-L-TyrNH₂ (Chen et al., 1991). The re-refined coordinates for the entire crystal structure have been submitted as 2bn2 to the Protein Data Bank. The sole significant differences between the coordinates used for modeling and the slightly more refined structure 2bn2 are in the distances between the amide N position of Asn48 and positions CD₂ and CE₂ of the tyrosine ring, which are, respectively, 0.7 and 0.5 Å greater in 2bn2 than in the coordinates available at the time of modeling. Other distances are within 0.1–0.3 Å of those in 2bn2, with almost all differences <0.1 Å. Since the variation between subunits of distances to the amide of Asn48 typically exceed 0.7 Å, both in the refined 2bn2 structure and in that of the oxytocin complex 1NPO (e.g., Table 4), and because these particular distances have little influence on the modeling, their change upon structure refinement is not considered significant. It is relevant that, of the four subunits that comprise the asymmetric unit in the dipeptide complex, subunit A contains the tightest binding pocket and is therefore the most sensitive indicator of potential steric hindrance to binding. Any significant differences found on comparison of results for subunit A with those for the other subunits or for the oxytocin complex are cited in Results.

The geometry of the binding pocket was determined by an extension of the Lee and Richards (1971) method for measuring solvent accessibility, using a probe of 1.2 Å radius. (Occasional calculations as indicated used the radius of a water molecule (1.4 Å).) The X-ray coordinates were transformed by rotations to approximately orient the Tyr ring of the ligand in the *x,z* plane with a line from Tyr CG to Tyr CZ parallel to the *z*-axis. Circles (extended atomic projections, labeled in capitals in Fig. 6), representing projection of atoms on to the *x,y* plane, with radii calculated as the sum of van der Waals and probe radii, were computed for sections through the domain of the cavity at 0.25 Å intervals along the *z*-axis. For these calculations, atomic van der Waals radii given by Bondi (referenced by Lee & Richards, 1971) were used.

To illustrate the method, Figure 6 is a section near the position occupied by CZ of the Tyr ring of Phe-TyrNH₂. The accessible domain contour (central black cavity) defines the center of a probe as it rolls along the cavity surface without penetrating the van der Waals space of another atom (Lee & Richards, 1971). The actual pocket surface is thus located at a position that is always the radius of the probe away from the accessible domain. A portion of this surface envelope must be on arcs of circles (referred to here as closest contact circles and labeled with small letters) located the probe radius away from the projections labeled with capital letters. The remaining part of the cavity envelope is located on the probe surface as it occupies positions between two closest contact circles

Fig. 6. In this slice through the binding cavity, the seven extended atomic projections surrounding the accessible domain (dark central region) are labeled A–G. Two circles containing points of closest contact with the probe (a and g) are constructed to be the probe radius away from the circumference of A and G, respectively. The dashed circle has the probe radius centered at that intersection of circles A and G bounding the accessible area and represents the position that the probe occupies in the crevice between a and g.

(Fig. 6). For this slice, the seven probe circles and seven closest contact circles form 14 contacts as shown in Figure 7. The x, y pairs defining the cavity envelope are computed for each circle between the angle of the first and second contact in steps of 0.01 radians. A plot of these points delineates the cavity surface at this position as demonstrated by the solid curve in Figure 7.

Modeling of the effects of substitutions in peptide position 2

Molecular mechanics calculations were made using HyperChem (HC) for the PC developed by Hypercube Inc. (Gainesville, Florida) and distributed by license agreement to Autodesk Inc. (San Rafael, California) (1992). Results were surveyed for reasonableness of fit with a program written in FoxPro 2.0 that printed out the distances below some cutoff value (usually 4 Å) between all ligand atoms and NP atoms. The van der Waals energy was estimated for each such atom pair by using a 6-12 Lennard–Jones potential with parameters as given by HC. Molecular mechanics calculations were done using the AMBER 3 force field.

The goal of these calculations was to determine whether substitutions for the Tyr ring would fit in the binding pocket and, if so, to estimate their van der Waals contacts to NP. The following steps were involved: (1) Position the substitution in the binding pocket. (2) Minimize the energy of residue 2 as a starting point. The conjugate gradient method with a convergence limit for the gradient of 10^{-5} kcal/(Å mol) is used. (3) If the fit is unsatisfactory after minimization, run molecular dynamics simulations for various times at 300 K (or higher to overcome potential barriers, followed by annealing back to 300 K). Minimize the energy again. (4) Use the FoxPro program to search for major violations of van der Waals distances in the minimized structures. (5) Determine the energy difference of atoms in the pocket before and after minimi-

Fig. 7. Plot of the 1,318 points (heavy solid curve) computed for the probe (circles with small dashes) and closest contact circles (dashed circles labeled a–g, corresponding to projections A–G in Fig. 5) as the probe rolls around the pocket. The envelope length is 16.2 Å (sum of distances between nearest neighbor points) and the bounded area is 17.8 Å² (sum of average adjacent y values times the corresponding Δx).

zation by single point calculations on the potential surface. This calculation is made after deleting all other atoms of both NP and the ligand. Since only the atomic coordinates are involved, this gives a measure of the strain imposed by nonideal bond distances and angles. During all other molecular mechanics calculations, the atomic coordinates of NP and the Phe in position 1 of the ligand remain fixed at their positions determined by the X-ray structure.

When the above procedures are applied as a control to the insertion of the tyrosine ring in the binding pocket (using Tyr-NH₂ as residue 2 and including H atoms), energy minimization, starting with the X-ray coordinates, indicates a ring strain of 0.75 kcal/mol. This value is to be compared with values reported in Discussion for Tyr substitutions. (The inclusion of H atoms in the calculations was found to be essential for realistic calculations of conformational strain in Cha, and was adopted for all residue 2 side chains.) Substitution of a cyclohexane ring for the Tyr ring was complex because AMBER has no template for Cha. We first assigned the cyclohexane carbons using the same coordinates as Tyr. The OH and ring hydrogens were removed, the ring changed to single bonds and the carbon types to tetrahedral, and the H atoms added back. Atomic charges left over from the Tyr template were edited to be 0 in the AMBER input file. Molecular mechanics then ran smoothly using the AMBER force field.

Supplementary material in Electronic Appendix

Two representations of the neurophysin pocket that binds Tyr2 of the hormones are submitted on diskette as Kinemages entitled nphysin.kin and supplied as a Microsoft Word text document.

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References

- Balaram P, Bothner-By AA, Breslow E. 1973. Nuclear magnetic resonance studies of the interaction of peptides and hormones with bovine neurophysin. *Biochemistry* 12:4695–4704.
- Blumenstein M, Hrubby VJ, Viswanatha V. 1979. Investigation of the interactions of oxytocin with neurophysins at low pH using carbon-13 nuclear magnetic resonance and carbon-13 labeled hormones. *Biochemistry* 18:3552–3557.
- Blumenstein M, Hrubby VJ, Viswanatha V. 1980. The tyrosine ring of oxytocin undergoes hindered rotation when the hormone is bound to neurophysin. *Biochem Biophys Res Commun* 94:431–437.
- Blumenstein M, Hrubby VJ, Viswanatha V. 1981. Dynamic properties of aromatic rings of oxytocin and arginine vasopressin on hormone binding to neurophysins determined by ^{13}C and ^{19}F NMR. In: Rich DH, Gross E, eds. *Peptides, synthesis, structure, function*. Proc. 7th American Peptide Symposium. pp 363–365.
- Breslow E. 1984. Neurophysin: Biology and chemistry of its interactions. In: Cantin M, ed. *Cell biology of the secretory process*. Basel, Switzerland: Karger. pp 276–308.
- Breslow E, Aanning HL, Abrash L, Schmir M. 1971. Physical and chemical properties of the bovine neurophysins. *J Biol Chem* 246:5179–5188.
- Breslow E, Abrash L. 1966. The binding of oxytocin and oxytocin analogs by purified bovine neurophysins. *Proc Natl Acad Sci USA* 56:640–646.
- Breslow E, Burman S. 1990. Molecular, thermodynamic and biological aspects of recognition and function in neurophysin-hormone systems: A model system for the analysis of protein-peptide interactions. *Adv Enzymol* 63:1–67.
- Breslow E, Gargiulo P. 1977. Effect of low pH on neurophysin-peptide interactions: Implications for the stability of the amino-carboxylate salt bridge. *Biochemistry* 16:3397–3406.
- Breslow E, LaBorde T, Bamezai S, Scarlata S. 1991. Binding and fluorescence studies of the relationship between neurophysin-peptide interaction and neurophysin self-association: An allosteric system exhibiting minimal cooperativity. *Biochemistry* 30:7990–8000.
- Breslow E, Sardana V, Deeb R, Barbar E, Peyton DH. 1995. NMR behavior of the aromatic protons of bovine neurophysin-I and its peptide complexes: Implications for solution structure and for function. *Biochemistry* 34:2137–2147.
- Breslow E, Stahl GL, Walter R. 1980. L-2-Hydroxy-3-mercaptopropionic acid)-oxytocin: Circular dichroism studies of conformation and interaction with neurophysin. *Int J Pept Protein Res* 15:314–322.
- Breslow E, Weis J, Menendez-Botet CJ. 1973. Small peptides as analogs of oxytocin and vasopressin in their interactions with bovine neurophysin-II. *Biochemistry* 12:4644–4653.
- Burley SK, Petsko GA. 1988. Weakly polar interactions in proteins. *Adv Protein Chem* 39:125–189.
- Camier M, Alazard R, Cohen P, Pradelles P, Morgat JL, Fromageot P. 1973. Hormonal interactions at the molecular level. A study of oxytocin and vasopressin binding to bovine neurophysins. *Eur J Biochem* 32:207–214.
- Chaiken IM, Tamaoki H, Brownstein MJ, Gainer H. 1983. Onset of neurophysin self-association upon neurophysin/neuropeptide hormone precursor biosynthesis. *FEBS Lett* 164:361–365.
- Chauvet MT, Hurpet D, Chauvet J, Acher R. 1983. Identification of human neurophysins: Complete amino acid sequences of MSEL- and VLDV-neurophysins. *Proc Natl Acad Sci USA* 80:2839–2843.
- Chen L, Rose JP, Breslow E, Yang D, Chang WR, Furey WF Jr, Sax M, Wang BC. 1991. Crystal structure of a bovine neurophysin II dipeptide complex at 2.8 Å determined from the single-wavelength anomalous scattering signal of an incorporated iodine atom. *Proc Natl Acad Sci USA* 88:4240–4244.
- Dougherty DA. 1996. Cation- π interactions in chemistry and biology: A new view of benzene, Phe, Tyr and Trp. *Science* 271:163–168.
- Eisenberg D, McLachlan AD. 1986. Solvation energy in protein folding and binding. *Nature* 319:199–203.
- Eriksson AE, Baase WA, Zhang XJ, Heinz DW, Blaber M, Baldwin EP, Matthews BW. 1992. Response of a protein structure to cavity-creating mutations and its relation to the hydrophobic effect. *Science* 255:178–183.
- Fauchere JL, Pliska V. 1983. Hydrophobic parameters of amino acid side chains from the partitioning of N-acetyl-amino-acid amides. *Eur J Med Chem* 18:369–375.
- Freist W, Sternbach H, Cramer F. 1996. Phenylalanyl-tRNA synthetase from yeast and its discrimination of 19 amino acids in aminoacylation of tRNA(Phe)-D-D-A and tRNA(Phe)-C-C-aA(3'NH₂). *Eur J Biochem* 240:526–531.
- Hummel JP, Dreyer WJ. 1962. Measurement of protein binding phenomena by gel filtration. *Biophys J* 63:530–532.
- Lee B. 1993. Estimation of the maximum change in stability of globular proteins upon mutation of a hydrophobic residue to another of smaller size. *Protein Sci* 2:733–738.
- Lee B, Richards FM. 1971. The interpretation of protein structures; estimation of static accessibility. *J Mol Biol* 55:379–400.
- Live DH, Cowburn D, Breslow E. 1987. Binding of oxytocin and 8-arginine-vasopressin to neurophysin studied by ^{15}N NMR using magnetization transfer and indirect detection via protons. *Biochemistry* 26:6415–6422.
- Makhatadze GI, Privalov PL. 1994. Energetics of interactions of aromatic hydrocarbons with water. *Biophys Chem* 50:285–291.
- Morton AM, Base WA, Matthews BW. 1995. Energetic origins of specificity of ligand binding in an interior nonpolar cavity of T4 lysozyme. *Biochemistry* 34:8564–8575.
- Nicolas P, Wolff J, Camier M, DiBello C, Cohen P. 1978. Importance of neurophysin dimer and of tyrosine-49 in the binding of neurohypophyseal peptides. *J Biol Chem* 253:2633–2639.
- Ooi T, Oobatake M, Nemethy G, Scheraga HA. 1987. Accessible surface areas as a measure of the thermodynamic parameters of hydration of peptides. *Proc Natl Acad Sci* 84:3086–3090.
- Radzicka A, Wolfenden R. 1988. Comparing the polarities of the amino acids; side-chain distribution coefficients between the vapor phase, cyclohexane, 1-octanol, and neutral aqueous solution. *Biochemistry* 27:1664–1670.
- Reid KSC, Lindley PF, Thornton JM. 1985. Sulphur-aromatic interactions in proteins. *FEBS Lett* 190:209–213.
- Rose JP, Wu CK, Hsiao CD, Breslow E, Wang BC. 1996. Crystal structure of the neurophysin-oxytocin complex. *Nature Struct Biol* 3:163–169.
- Sharp KA, Nicholls A, Friedman R, Honig B. 1991. Extracting hydrophobic free energies from experimental data: Relationship to protein folding and theoretical models. *Biochemistry* 30:9686–9697.
- Sur S, Rabbani LD, Libman L, Breslow E. 1979. Fluorescence studies of native and modified neurophysins: Effects of peptides and pH. *Biochemistry* 18:1026–1036.
- Thomas KA, Smith GM, Thomas TB, Feldman RJ. 1982. Electronic distributions within phenylalanine aromatic rings are reflected by the three-dimensional oxygen atom environments. *Proc Natl Acad Sci USA* 79:4843–4847.
- Wang J, Breslow E, Sykes BD. 1996. Differential binding of desmopressin and vasopressin to neurophysin-II. *J Biol Chem* 271:31354–31359.
- Whittaker BA, Allewell NM, Carlson J, Breslow E. 1985. Enthalpies of ligand binding and subunit interactions in bovine neurophysins. *Biochemistry* 24:2782–2790.
- Zheng C, Cahill S, Breslow E. 1996. The behavior of the active site salt bridge of bovine neurophysins as monitored by ^{15}N NMR spectroscopy and chemical substitution. Relationship to biochemical properties. *Biochemistry* 35:11763–11772.