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†

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ABSTRACT: The active site of liganded neurophysin contains a salt bridge that involves the side chains of Arg-8 and Glu-47 of the protein and the α-amino group of bound hormone or related peptide. The extent to which the Arg-8−Glu-47 salt bridge persists in the absence of peptide, or to which the environment of Arg-8 in the unliganded state differs in monomers and dimers, is relevant to an understanding of allosteric mechanism in this system. In the present study, the behavior of the salt bridge was investigated by ^{15}N NMR and chemical replacement of Arg-8. Bovine neurophysin-I was converted to its des 1−8 derivative, and Arg-8 was replaced by ^{15}N-substituted Arg or by other residues using chemical semisynthesis. The relative abilities of different amino acids to restore peptide affinity to the des 1−8 protein were in good accord with the view of the salt bridge in the liganded state obtained from crystals of bovine neurophysin-II complexes. In the unliganded state, comparison of the ^{15}N and proton NMR signals from Arg-8 with those in smaller arginine systems suggested the absence of significant interactions between the guanidinium of Arg-8 and Glu-47 or between the amino terminal region of Arg-8 and other elements of the protein. No evidence of a difference in Arg-8 environment between unliganded monomers and dimers was found. Marked spectral changes accompanying the binding of oxytocin indicated changes in the environment of both the side chain and amino terminal region of Arg-8. The NMR results were in good agreement with a recently emerging comparison of bovine neurophysin-II derivatives in the liganded and unliganded states, with the notable exception of the extent of salt bridge formation in the unliganded state. The results are shown to be consistent with, and to help explain, significant differences between the two bovine neurophysins in the susceptibility to tryptic cleavage at Arg-8 in the unliganded state and in the pH dependence of peptide binding and additionally constrain potential allosteric mechanisms underlying neurophysin ligand-facilitated dimerization.

The binding of posterior pituitary hormones and related peptides to neurophysin involves formation of a salt bridge between the protonated α-amino group of the peptide and a side chain carboxyl group from the protein. This was deduced early from solution studies (Stouffer et al., 1963; Ginsburg & Ireland, 1964) and demonstrated conclusively by the crystal structure of a dipeptide complex of bovine neurophysin-II (Chen et al., 1991). This crystal structure, which was more recently shown to be essentially the same as that of the oxytocin complex (Rose et al., 1991, 1996), identified the carboxyl group as that of the side chain of Glu-47 and demonstrated that the salt bridge was complex, also involving interactions between the Glu-47 carboxyl and the guanidinium side chain of Arg-8 of the protein; participation of Arg-8 in the salt bridge helped to explain the large decrease in binding affinity previously found to be associated with its chemical modification or excision (Abercrombie et al., 1982; Breslow et al., 1982; Sardana & Breslow, 1984). The multiple interactions involved in the neurophysin salt bridge are in some respects similar to the interactions in hemoglobin among the α-amino group of an α-chain, a side chain carboxyl and a guanidinium side chain, although the latter also involve a stabilizing chloride ion for which there is no evidence in the case of neurophysin complexes (Baldwin, 1976).

Although the structure of the liganded state of a neurophysin has been solved, relatively little information has been available about the structure of the unliganded state. Dimerization of the liganded state is strongly enhanced relative to that of the unliganded state (Nicolas et al., 1980). This enhanced dimerization, which is the thermodynamic correlate of stronger binding to the dimer than to the monomer, represents long-range communication between the subunit interface and the binding site (Chen et al., 1991) and might be mediated by the demonstrated conformational differences between unliganded monomers and dimers, between unliganded and liganded dimers, or both (Breslow & Burman, 1990; Breslow et al., 1991, 1992). Two lines of evidence have suggested a role for Arg-8, and accordingly of the salt bridge between Arg-8 and Glu-47, in the allosteric mechanism. First, under appropriate ionic conditions, excision of Arg-8 altered the linkage between peptide binding and dimerization (Huang et al., 1993). Second, NMR studies indicated that the environment of Tyr-49, a residue adjacent to the peptide-binding site, was altered by the excision of Arg-8 in the unliganded dimer but not in the unliganded

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monomer (Peyton et al., 1986; Breslow et al., 1992). One possible interpretation of these results is that interactions of Arg-8 differ significantly in unliganded monomers and dimers. This study explores the interactions of Arg-8 in unliganded monomers and dimers and compares these with the liganded state.

All studies of the effects of cleavage of Arg-8 have historically been carried out using bovine neurophysin-I, which exhibits >75% homology to bovine neurophysin-II, with which the structure of the liganded state was determined crystallographically. The two neurophysins are virtually identical in binding and allosteric properties (e.g., Breslow & Burman, 1990). Selective cleavage of the 1−8 sequence in BNP-I[1] can be accomplished by limited tryptic digestion (Abercrombie et al., 1982; Breslow et al., 1982). Our approach to studying the behavior of Arg-8 was therefore to prepare des 1−7 neurophysin in which Arg-8 nitrogen atoms were uniformly substituted with ^{15}N and to examine the behavior of ^{15}N resonances and their associated protons by NMR in the absence and presence of peptide ligands. The procedure involved excision of the 1−8 sequence from BNP-I and reattachment of Arg-8 by chemical semisynthesis. Extension of these studies to BNP-II was not feasible, for reasons described below that appear to reflect significant differences between the two proteins in the unliganded state. This approach also allowed the effects of replacement of Arg-8 by other residues to be investigated, thereby providing further insights into Arg-8 function.

METHODS

Chemicals and Enzymes. *N*-α-Boc-amino acids, Phe-Phe-NH_2, Phe-Tyr-NH_2, Arg-Gly-NH_2, and oxytocin were purchased from Bachem Bioscience Inc. L-Arginine hydrochloride (98% U-$^{13}C_6$ and U-$^{15}N_4$) was obtained from Cambridge Isotope Laboratories. Both Arg-Gly-NH_2 and *Arg were converted to their *N*-α-Boc derivatives by the general method of Itoh et al. (1975) using Boc-On (Aldrich). Trypsin (diphenylcarbamyl chloride treated), type XI, was obtained from Sigma. All amino acids were in the L-configuration unless indicated otherwise.

Preparation of Native and Modified Neurophysins. Bovine NP-I and -II were purified as described elsewhere (Breslow et al., 1971; Virmani-Sardana & Breslow, 1983). BNP-II was converted to its des 1−6 derivative by the procedure of Zheng et al. (manuscript in preparation). Conversion of proteins to their purified mononitrated derivatives used the procedure of Sardana et al. (1987).

BNP-I was prepared for semisynthesis by first blocking its two Lys residues (Lys-18 and -59) by exhaustive acetimidation with ethyl acetimidate as described elsewhere (Huang et al., 1993). The crude acetimidated product was purifed by affinity chromatography using the procedure of Rabbani et al. (1982) to remove inactive protein. Des 1−8 [$N^{\epsilon 18,59-diAcet}$] BNP-I was prepared by limited trypsin digestion (75 min digestion at room temperature with 1% trypsin by weight at pH 7.3 in 0.05 M Tris buffer) and followed by affinity chromatography (Peyton et al., 1986). The final yield of the acetimidated des 1−8 derivative relative to unmodified BNP-I was 35−45%.

Semisynthesis of Des 1−7 Acetimidated BNP-I Derivatives from Acetimidated Des 1−8 BNP-I. To prepare des 1−7 acetimidated BNP-I, Boc-Arg·HCl (100 mg) was dissolved in 250 μL of DMF containing *N*-hydroxysuccinimide (16.5 mg). The solution was placed in an ice bath, and 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride (24.7 mg) was added. After the solution was stirred overnight at 4 °C, formation of the succinimide ester was judged complete by TLC and the active ester was used in the next step without isolation. To the active ester solution, des 1−8 acetimidated BNP-I (20 mg), *N*-methylmorpholine (50 μL), and DMF (500 μL) were added. After 4 days of stirring at 4 °C, the reaction mixture was diluted with 20 mL of water and dialyzed against water to remove excess reagents. The protein was lyophilized and then treated with 1 M NH_2OH at pH 10 for 1 h. This step reversed acylation at Ser (or Thr) hydroxyl groups (see Results). The treated protein was dialyzed against water and purified by affinity chromatography. The des 1−8 protein eluted from the affinity column at pH 6.2 immediately after the void volume (Peyton et al., 1986). The Boc des 1−7 acetimidated protein, like native BNP-I (Rabbani et al., 1982), elutes when the pH is lowered to 2.1. The recovered Boc des 1−7 acetimidated BNP-I was dialyzed against water and lyophilized. On nondenaturing PAGE at a running pH of 9.5 (e.g., Huang et al., 1993), it migrated as a single band with the expected reduced mobility to the anode relative to acetimidated des 1−8 BNP-I. (Differences in a single charge are readily observed in neurophysin electrophoresis patterns [Rabbani et al., 1982].) The final yield of the active semisynthetic Boc des 1−7 derivative relative to the des 1−8 protein was 15%. For removal of the Boc group, the protein was treated with trifluoroacetic acid (TFA) at 0 °C for 30 min. The TFA was quickly removed by lyophilization, and the protein was re-purified by affinity chromatography. This procedure reduced the overall yield of active protein to 5−10%.

Preparation of the des 1−7 derivative uniformly labeled with ^{13}C and ^{15}N at Arg-8 was accomplished by the same methodology, beginning with the Boc derivative of the isotope-enriched amino acid. The synthesis of derivatives in which Arg-8 was substituted by L-His, L-Cit, D-Cit, L-Tyr, L-Phe, L-Trp, and L-Gln was also initiated by the same methodology using the corresponding Boc amino acids. The products resulting from the coupling of L-His and L- or D-Cit behaved similarly to the des 1−7 derivative on affinity chromatography and were therefore also purified using the same methodology. N-Terminal sequences of derivatives with Arg, His, or Cit in position 8 were confirmed by automated Edman degradation (e.g., Huang et al., 1993). The other amino acids gave no products that were bound to the affinity column more strongly than the des 1−8 protein and were not further investigated.

Determination of Peptide-Binding Constants, Dimerization Constants, and Related Spectroscopic Studies. Binding

[1] Abbreviations: NP, neurophysin; BNP-I, bovine neurophysin-I; BNP-II, bovine neurophysin-II; *Arg, L-arginine uniformly labeled (98% enrichment) with ^{13}C and ^{15}N; Boc-*Arg, the N-α-Boc derivative of *Arg; *des 1−7 BNP-I, des 1−7 BNP-I acetimidated at its two Lys residues and containing *Arg in position 8; Boc-*des 1−7 BNP-I, the *N*-α-Boc derivative of *des 1−7 BNP-I; Boc, *tert*-butoxycarbonyl; Boc-Arg-Gly-NH_2, the *N*-α-Boc derivative of L-arginylglycine amide; Phe-Phe-NH_2, L-phenylalanyl-L-phenylalanine amide; Phe-Tyr-NH_2, L-phenylalanyl-L-tyrosine amide; Cit, citrulline; CD, circular dichroism; DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate; NOE, nuclear Overhauser effect. Nomenclature used for the guanidinium side chain: N^{ϵ}, imino nitrogen; N^{η}, terminal nitrogens; C^{ζ}, guanidinium carbon.

affinities of the des 1−7 derivatives for Phe-Phe-NH_2 were monitored by fluorescence as described elsewhere (Sardana & Breslow, 1984) using a Kontron SFM 25 spectrofluorometer. Binding studies were carried out in 0.1 M sodium phosphate buffer at pH 6.2 and a protein concentration of $\sim 5 \times 10^{-5}$ M.

Comparative studies of the effects of pH on binding by bovine neurophysins-I and -II involved the mononitrated derivatives of these proteins; binding was followed by changes in the circular dichroism (CD) spectrum of the nitrotyrosine chromophore as described elsewhere (Breslow & Gargiulo, 1977). A Jasco J-710 spectropolarimeter was used for these studies and for parallel studies of the pH dependence of nitrotyrosine ellipticity. Studies were carried out in 0.1 M sodium phosphate buffer at pH 6.2 and 3.5 and a protein concentration of 5×10^{-5} M. Binding constants determined under these conditions were corrected for the relative concentrations of monomer and dimer in the unliganded state at the two pH values as determined from proton NMR spectra of BNP-I and -II in the same buffer system at the two pH values. For BNP-I, the relative intensities of α-proton signals at ~6.4 ppm (dimer) and 6.2 ppm (monomer) to each other or to Tyr 3,5 ring proton signals allowed facile determination of dimerization equilibria as described elsewhere (Breslow et al., 1992). BNP-II spectra can be shown to be complicated by the presence of signals that overlap the 6.2 ppm monomer resonance at low pH. Accordingly, the fractional content of dimer in BNP-II was determined from the intensities relative to the Tyr 3,5 ring proton signal of dimer signals at both 6.4 ppm and 7.57 ppm (Breslow et al., 1992). Dimerization constants for BNP-II calculated by this method were in excellent agreement with published values obtained by ultracentrifugation (Nicolas et al., 1980). Reported binding constants to the dimer were calculated from observed binding constants, assuming that binding to the monomer, which occurs with less than 1/10 of the affinity of binding to dimer (Breslow et al., 1991), can be neglected. This assumption has only trivial impact on derived pK_a values. If the monomer is assumed to bind with as much as 1/10 of the affinity of dimer, derived pK values are reduced by 0.04 and 0.05 pH units for BNP-I and -II, respectively.

CD titrations of the effect of pH on the ellipticity of the nitrotyrosine signal in mononitrated neurophysins were similarly carried out in 0.1 M sodium phosphate buffer at a protein concentration of 5×10^{-5} M. The nitrotyrosine signal of unliganded nitrated NP is strongly pH-dependent between pH 6.2 and 3, reflecting titration of a carboxyl group (Breslow & Gargiulo, 1977). The data were fitted to obtain approximate pK_a values for the different proteins as described elsewhere (Breslow & Gargiulo, 1977).

NMR Studies. All NMR samples were dissolved in 90% H_2O/10% D_2O and were titrated with NaOH or HCl to the desired pH. NMR experiments were performed at 5 °C on a Bruker DMX600 spectrometer unless indicated otherwise. ^1H spectra were obtained with 128 scans of 4K points. 1D ^{13}C spectra were obtained using proton and ^{15}N decoupling. $^1H\{^{15}N\}$ HMQC spectra were obtained with either 32 or 256 scans of 2K t2 points and 128 t1 points, with sweep widths of 8000 and 3600 Hz for ^1H and ^{15}N, respectively. The water signal in each experiment was suppressed using a 3−9−19 pulse sequence with a *z*-field gradient (the watergate method) (Piotto et al., 1992). $^1H\{^{15}N\}$ HMQC spectra were obtained at the following concentrations: *Arg, 56 mM; Boc-*Arg, 10 mM; Boc-Arg-Gly-NH_2, 41 mM; Boc *des 1−7 BNP-I, 1 mM; *des 1−7 BNP-I, 0.5 mM. ^1H chemical shifts are reported relative to an external DSS standard (DSS chemical shift at 0.0 ppm). The zero position of the ^{15}N scale is given by the ratio of the frequencies of ^{15}N and ^1H (e.g., Live et al., 1984). ^{13}C data were used principally to aid in the initial small molecule resonance assignments and for line width studies.

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                    10                  20                  30
BNP-II AMSDLE LRQCLPCGPGGKGRCFGPSICCGDELGCF
BNP-I  ---V  L------D V---T----------------------------------------------------

                    40                  50                  60                  70
BNP-II VGTAEALRCQEENYLPSPCQSGQKPCGSGGRCAAA
BNP-I  ----------------------------------------------------------------------

                    80                  90
BNP-II GICCNDESCVTEPECREGIVGFPRRV
BNP-I  ----------SPDG----HED---A---DPE AA--- SQ
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FIGURE 1: Amino acid sequences of bovine neurophysins-I and -II (Chauvet et al., 1979). The sequence of BNP-I is shown only through residue 92; residue 93 is not present in our preparations. Proteins are identical except where substitutions are listed.

Table 1: Relative Susceptibilities of Bovine Neurophysins-I and -II to Tryptic Cleavage after Arg-8[a]

	products (%)			
protein	des 1−8 NP	native NP	des 94−95 (BNP-II)	other[b]
BNP-I (unliganded)	66	29		5
BNP-I (liganded)	0	95		5
BNP-II (unliganded)[c]	0	0	100	0
BNP-II (liganded)	0	0	100	0

[a] Conditions: Tris buffer (pH 7.3, 0.05 M) in 0.16 M KCl, 75 min digestion at room temperature, 1% trypsin by weight. For studies of the liganded state, Phe-Tyr-NH_2 (4 mg/mL) was included in the digestion buffer. All products were quantitated by weight, with the exception of des 94−95 BNP-II, which was demonstrated by nondenaturing PAGE, affinity chromatography, and quantitative amino acid analysis to be the sole product of BNP-II digestion under these conditions. [b] Represents protein with clips at internal Arg/Lys residues, together with probable clips at Arg-8. [c] Identical results were obtained with unliganded BNP-II when the trypsin concentration was increased to 2% by weight.

RESULTS

Different Susceptibilities of Bovine Neurophysins-I and -II to Tryptic Cleavage at Arg-8. Figure 1 compares the amino acid sequences of bovine neurophysins-I and -II. In the absence of peptide ligands, BNP-I can be relatively easily converted by trypsin to its des 1−8 derivative (Table 1). This derivative has no internal points of tryptic cleavage, although internal cleavage by attack at Lys-18 and several Arg residues can also occur as an overlapping process, yielding derivatives with no binding ability which are separated from the uninterrupted des 1−8 derivative by affinity chromatography (Peyton et al., 1986). Under standard digestion conditions (see Methods), more than 60% of the protein is recovered as the des 1−8 derivative; the remainder is principally native protein, but a low percentage of inactive protein containing clips at internal positions is also recovered (Table 1). Under the same conditions, but in the presence of peptide ligand, no cleavage occurs and all of the protein is recovered as native protein (Table 1).

Unliganded BNP-II is markedly less susceptible than unliganded BNP-I to tryptic cleavage at Arg-8. Under the

same digestion conditions, all protein exhibits unmodified behavior on affinity chromatography. Analysis of the trypsin-exposed protein by electrophoresis and amino acid analysis confirms the lack of cleavage at Arg-8 and indicates complete tryptic cleavage at Arg-93, a cleavage previously shown not to alter NP properties (Sardana & Breslow, 1984). This behavior is unaltered in the presence of peptide (Table 1).

The resistance of the Arg-8−Gln-9 bond in unliganded BNP-II to tryptic cleavage under the above conditions (Tris buffer, pH 7.3) is paralleled by the resistance of the Arg-86−Glu-87 bond to cleavage. If digestion is carried out in the absence of peptide in borate buffer (pH 8.0), then different results are obtained. Under these conditions, the initial product formed remains the des 94−95 derivative, but product can be identified in ∼20% yield that represents cleavage at both Arg-8 and Arg-86 as described earlier (Breslow et al., 1982). However, no product representing cleavage at Arg-8 without cleavage at Arg-86 has yet been isolated. The results suggest that cleavage at Arg-86 is necessary to sensitize the Arg-8 bond, or vice versa, but in any event preclude preparation of a des 1−8 derivative of BNP-II that does not also have a significant C-terminal deletion. For this reason, all of the studies reported utilized the des 1−8 derivative of BNP-I.

Development of Semisynthesis Methodology. Bovine NP-I was acetimidated at its two Lys residues to block coupling to ϵ-amino groups and was then converted to the corresponding des 1−8 derivative. Coupling of Boc-protected amino acids to the α-amino of Thr-9 of acetimidated des 1−8 BNP-I proceeded with only 15% efficiency under the conditions used, despite the utilization of a very large excess of the activated Boc amino acid (see Methods). The low efficiency reflected the inaccessibility of the α-amino group; for example, coupling to the α-amino group of Thr-9 did not occur at all under conditions giving highly efficient coupling to the accessible α-amino group of native BNP-II (data not shown). The necessity of using high concentrations of the activated Boc amino acid to drive coupling at Thr-9 led to side reactions giving acylation of protein OH groups, probably analogous to the previously demonstrated acylation of neurophysin Ser residues by succinic anhydride (Huang et al., 1993). These side reactions, which could be shown to proceed more efficiently than coupling to the Thr-9 amino group, were effectively reversed after coupling by treatment with hydroxylamine, as also used earlier (Huang et al., 1993).

Binding Properties of Semisynthetic Des 1−7 Derivatives of Bovine NP-I. Selected Boc L-amino acids were attached to the amino terminus of residue 9 by semisynthesis; for Boc-citrulline both L- and D-isomers were individually coupled. The resultant derivatives were subjected to affinity chromatography and given an initial classification according to binding competence by this method (Table 2). The binding affinities of binding-competent Boc des 1−7 derivatives for Phe-Phe-NH_2, which binds to the hormone-binding site (e.g., Breslow & Burman, 1990), were quantitatively determined by fluorescence (see Methods) and compared with that of native BNP-I, its acetimidated derivative, and acetimidated des 1−8 BNP-I. The affinities of several derivatives were also determined after removal of the Boc group. The results are shown in Table 2. The data indicate that, under the assay conditions used, acetimidation had no effect on binding affinity, as reported elsewhere (e.g., Ando et al., 1987), and that (as with the native protein [Sardana & Breslow, 1984]) cleavage of the 1−8 sequence of the acetimidated protein reduced binding affinity to ∼2% of its initial value. The ability to bind to the affinity column was restored by the addition of Arg, His, or Cit in position 8 but not by Trp, Phe, Leu, Gln, or Tyr.

Table 2: Binding of Phe-Phe-NH_2 to Native and Modified Neurophysins[a]

protein	affinity column behavior[b]	K (M^{-1})	$\Delta G°$ (kcal/mol)
BNP-I	binding	8300	5.5
Ac-BNP-I	binding	7700	5.4
des 1−8 Ac-BNP-I	weak binding	180	3.2
des 1−7 Ac-BNP-I	binding	2800	4.8
L-Cit des 1−8 Ac-BNP-I	binding	1000	4.2
Boc des 1−7 Ac-BNP-I	binding	1000	4.2
Boc-L-Cit des 1−8 Ac-BNP-I	binding	470	3.7
Boc-D-Cit des 1−8 Ac-BNP-I	binding	400	3.7
Boc-His des 1−8 Ac-BNP-I	binding	720	4.0
Boc-Trp des 1−8 Ac-BNP-I	nonbinding, weak binding		
Boc-Phe des 1−8 Ac-BNP-I	nonbinding, weak binding		
Boc-Leu des 1−8 Ac-BNP-I	nonbinding, weak binding		
Boc-Gln des 1−8 Ac-BNP-I	nonbinding, weak binding		
Boc-Tyr des 1−8 Ac-BNP-I	nonbinding, weak binding		

[a] Conditions: pH 6.2, 0.1 M phosphate, 25 °C, protein concentration = 0.05 mM. [b] Binding properties are defined as follows: nonbinding, elutes in void volume of affinity column at pH 6.2; weak binding, elutes immediately after the void volume at pH 6.2; binding, retained on the column at pH 6.2 for >30 mL (6 column volumes) and elutes when the pH is lowered to 2.1 (Rabbani et al., 1982). The properties reported are those of the purified proteins except for the products of coupling that were not binding-competent (see Methods). For these products, the affinity column behavior reported is that of the crude coupling product. The presence of both nonbinding and weak binding components in this product probably signifies the presence of denatured protein.

The quantitative effects on binding affinity of addition at position 8 of Arg, His, or Cit are most clearly expressed as free energies (Table 2). Of the 2.2 kcal/mol in binding affinity lost upon cleavage of the 1−8 sequence, 1.6 kcal/mol is restored by the addition of unblocked L-Arg at position 8 and 1 kcal/mol is restored by the addition of unblocked L-citrulline. The fact that addition of Arg does not completely restore binding affinity indicates a weak contribution from other residues in the 1−8 sequence. The relative binding affinities of the Cit-8 and Arg-8 derivatives suggest that at least half of the effect of Arg is due to the hydrogen-bonding properties of its side chain (see Discussion).

The Boc derivatives bind more weakly than their unmodified counterparts, but the same trends are evident for Boc-Arg and Boc-L-Cit as with the unblocked amino acids. Also seen from the Boc des 1−7 derivatives is that His is almost as effective as Arg in restoring binding affinity and that, surprisingly, there is little difference between the D and L derivatives of citrulline. Also surprising is that the small increases in binding affinity produced by coupling of Boc-Cit lead to such a large change in affinity chromatography behavior (see legend, Table 2). A potential explanation of this is the restoration by functional substitutents at position 8 of the normal linkage between peptide binding and

Table 3: Chemical Shifts of Arg α-Amino/Amide Resonances in ^{15}N-Labeled Small Molecules and in Boc *Des 1−7 BNP-I Obtained from ^{1}H{^{15}N} HMQC Spectra[a]

		chemical shift (ppm)					
		α-amino		amide (trans)[b]		amide (cis)[a]	
	pH	^{15}N	^{1}H	^{15}N	^{1}H	^{15}N	^{1}H
Arg	4.8	41.2	7.55				
Boc-Arg	3.1			94.7	6.75	95.8	6.32
Boc-Arg	6.7			97.2	6.35	98.5	5.99
Boc-Arg-Gly-NH_2	3.8			93.3	6.95	94.0	6.50
Boc des 1−7 Ac-BNP-I	3.1			93.2	6.88	94.0	6.46
				91.8[c]	6.77[c]		
Boc des 1−7 Ac-BNP-I	6.7			93.4	6.85	94.3	6.44
Boc des 1−7 Ac-BNP-I							
+ 1 mM oxytocin	6.1			92.6	6.18	94.1	6.98
				93.2[d]	6.73[d]		
+ 2 mM oxytocin	6.1			92.6	6.18	94.2	6.98

[a] Conditions: temperature, 5 °C; concentrations of *Arg and Boc-*Arg, 10 mM; protein concentration, ∼1 mM. Where chemical shifts are not listed, the signals were not detected. [b] The predominant amide crosspeak in each 2D spectrum is assigned as the trans isomer. [c] Possible shoulder on main signal. [d] Signifies residual unbound protein, present as a minor component.

Table 4: Chemical Shifts of Arg Side Chain Resonances in ^{15}N-Labeled Small Molecules and in *Des 1−7 BNP-I Derivatives Obtained from ^{1}H{^{15}N} HMQC Spectra[a]

		chemical shift (ppm)					
		imino (N$^{\epsilon}$)		amino (N$^{\eta}$)		amino (N$^{\eta}$)	
	pH	^{15}N	^{1}H	^{15}N	^{1}H	^{15}N	^{1}H
Arg	4.8	85.2	6.95	73.8	6.62	71.2	6.62
					6.13		6.13
Boc-Arg	3.1	85.6	6.93	73.8	6.60	71.2	6.13
Boc-Arg	6.7						
Boc-Arg-Gly-NH_2	3.8	85.2	6.93	73.6	6.58	71.2	6.15
Boc des 1−7 Ac-BNP-I	3.1	85.3	6.90	74.0	6.60	71.5	6.18
Boc des 1−7 Ac-BNP-I	6.7	85.3	6.97	73.5	6.66	71.7	6.28
des 1−7 Ac-BNP-I[b]	6.1	84.8	7.04	73.9	6.72	71.5	6.27
Boc des 1−7 Ac-BNP-I							
+ 1 mM oxytocin	6.1	85.0	6.50				
		85.5[c]	6.99[c]				
+ 2 mM oxytocin	6.1	84.8	6.50				

[a] Temperature, 5 °C; concentrations of *Arg and Boc-*Arg, 10 mM; protein concentration, ∼1 mM. Where chemical shifts are not listed, the signals were not detected. [b] Data were obtained at 500 MHz. [c] Signifies residual unbound protein, present as a minor component.

dimerization and consequences of this for affinity column retention.

NMR Studies of ^{15}N- and ^{13}C-Substituted Arg and Boc-Arg. Evidence for Cis and Trans Boc-Arg Isomers. Deblocking procedures to remove Boc led to significant loss of binding-competent protein (see Methods). Accordingly, the Boc derivative of ^{15}N and ^{13}C-des 1−7 BNP-I was used for most studies. To ascertain that the Boc group did not alter the nature of the results, a small sample of the Boc derivative was also subsequently deblocked, repurified, and analyzed (vide infra). ^{1}H{^{15}N} HMQC spectra of the des 1−7 protein derivatives were therefore compared with the corresponding spectra of both *Arg and Boc-Arg derivatives, assignments for which are presented here (see Tables 3, and 4).

An initial result was the demonstration in Boc-*Arg of signals from the cis and trans isomers of the Boc-Arg amide bond. Figure 2 shows the ^{15}N and ^{13}C decoupled 1D proton

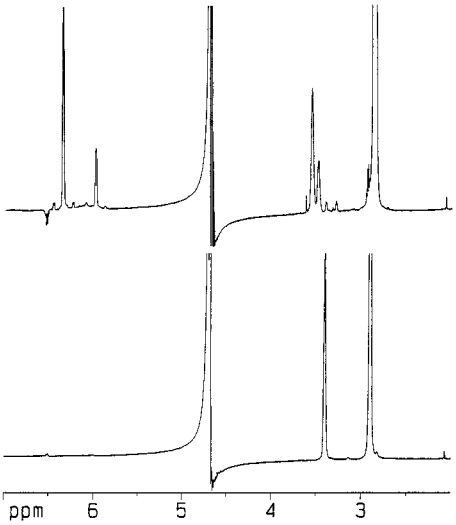

FIGURE 2: 1D proton spectra (^{15}N and ^{13}C decoupled) of *Arg (lower spectrum) and Boc-*Arg (upper spectrum) in the region of 2−7 ppm (temperature = 5 °C). The α-proton signal is located at ∼3.4 ppm in *Arg and at ∼3.5 and 3.6 ppm in Boc-*Arg. Peaks at 5.99 and 6.35 ppm in Boc-*Arg are the amide hydrogens (see text).

spectra of Boc-*Arg and *Arg at pH 6.7 and 5 °C. Of particular significance is a small peak in the spectrum of Boc-*Arg, located at ∼3.5 ppm adjacent to the main α-proton signal. This peak is absent in *Arg, and can be shown to be lost upon deblocking, indicating that it does not represent an irreversible alteration introduced upon addition of the Boc group (e.g., decarboxylation, conversion to ornithine, etc.). We assign the two signals in the α-proton region to the cis and trans Boc-*Arg isomers and tentatively assign the major component to the trans isomer, as calculated for analogous amides by Remko & Scheiner (1988). Consistent with assignment of these peaks to the different amide isomers, the two peaks coalesce with increasing temperature (data not shown). However, the specific cis and trans assignments could not be experimentally confirmed; the signals from the three Boc methyl groups were superimposable, precluding NOE assignments for an individual isomer. Also seen in Figure 2 are two peaks in the Boc-*Arg spectrum, absent in the *Arg spectrum, located at 6.35 and 5.99 ppm, respectively. The ratio of the intensities of these two peaks is similar to that of the two α-proton signals, and the two peaks are assigned on the same basis to the cis (minor component) and trans (major component) signals from the Boc amide hydrogen. Exchange between these two peaks is demonstrated with increasing temperature (data not shown).

The ^{1}H{^{15}N} HMQC spectra of Boc-*Arg at pH 6.7 and 3.1 are shown in Figure 3; assignments of the signals in these and other spectra are given in Tables 3 and 4. At pH 6.7, side chain protons exchange too rapidly for their signals to be detected under these conditions, and only the cis and trans amide signals are observable. At pH 3.1, the amide signals shift, showing the expected effect of titration of the α-carboxyl group. Three other signals, also seen in *Arg, are evident at pH 3.1. The strongest of these is assigned to the imino N (N$^{\epsilon}$) and its associated proton (Table 4). The weaker two peaks are assigned to the terminal amino nitrogens (N$^{\eta}$) of the side chain and their associated hydrogens (Table 4). Consistent with the terminal amino assignment, evidence of the chemical exchange of the terminal hydrogens resulting from rotation around the N$^{\epsilon}$−C$^{\zeta}$ bond (Yamazaki et al., 1995) is seen in *Arg spectra (Table 4). The side chain assign-

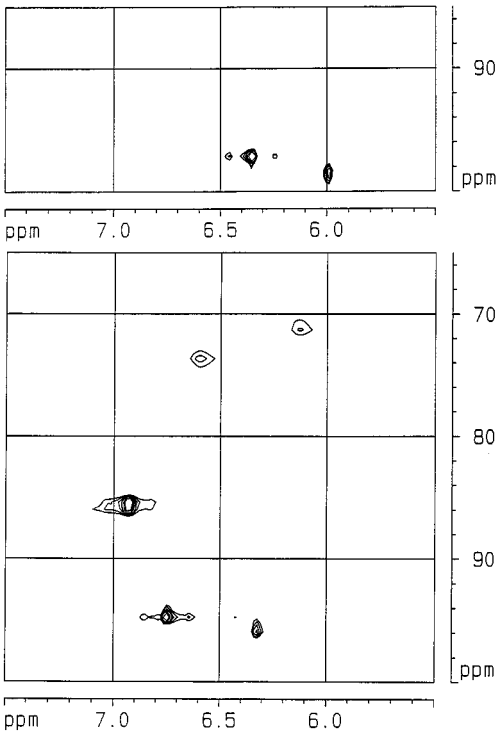

FIGURE 3: $^{1}H\{^{15}N\}$ HMQC spectra of Boc-*Arg (10 mM) at pH 3.1 (lower grid) and pH 6.7 (upper grid). The abscissa is the chemical shift scale for ^{1}H; chemical shifts for ^{15}N are given by the ordinate (temperature = 5 °C).

ments are consistent with Arg side chain assignments in other systems (e.g., Yamazaki et al., 1995). Side chain chemical shifts are not significantly altered by peptide bond formation, as can seen by the near identity of these shifts in Boc-Arg and in Boc-Arg-Gly-NH_2 (Table 4).

NMR Studies of ^{15}N- and ^{13}C-Substituted Arg and Boc-Arg in Des 1−7 Bovine NP-I. Figure 4 shows the $^{1}H\{^{15}N\}$ HMQC spectra of the Boc derivative of *des 1−7 BNP-I in the unliganded state (pH 6.7) and liganded state (pH 6.1); chemical shifts and assignments of these and related spectra are further given in Tables 3 and 4. In the unliganded state, ^{15}N signals assigned to the trans (major) and cis (minor) amide signals are identical to those in Boc-Arg-Gly-NH_2; small differences are seen in the proton signals, each 0.05−0.1 ppm further upfield in the protein than in the peptide. Peaks assigned to the side chain imino and amino groups are also almost identical in the unliganded protein and in Boc-Arg derivatives, but close inspection reveals subtle differences between the two cases. First, the protein side chain signals are detectable at both pH 6.7 and 3.1 (Table 4), in contrast to the absence of these signals in Boc-*Arg at the higher pH, suggesting a decreased rate of exchange with solvent in the case of the protein. Second, signals from the protein side chain amino protons are located 0.06 and 0.15 ppm downfield from those of Boc-*Arg at pH 6.7, while a maximum deviation of only 0.05 ppm downfield is present at pH 3.1. These results are consistent with an interaction between the Arg side chain amino group and a carboxylate side chain at pH 6.7 that is lost upon protonation of the carboxyl group, but the small magnitude of the chemical shift differences suggests that the interaction is weak (see Discussion). The differences in side chain ^{15}N chemical shifts between the unliganded protein and Boc-*Arg are relatively even smaller and more ambiguous.

Removal of the Boc group from the Boc *des 1−7 protein leads to loss of the amide resonances; α-amino group resonances were not observable due to exchange. Shifts in side chain signals resulting from deblocking were small and nonsystematic (Table 4) and are not viewed as significant (see Discussion).

Addition of oxytocin to the Boc *des 1−7 protein at pH 6 leads to major changes in all resonances (Figure 4 and Tables 3 and 4). The amide NH signals are the most affected, with the ^{15}N and ^{1}H of the trans (major) form shifting 0.8 and 0.67 ppm upfield, respectively, and the ^{1}H signal assigned to the cis (minor) isomer shifting over 0.5 ppm downfield upon hormone binding. The conclusion that the two amide isomers respond differently to binding remains unaltered even if the assignment of major and minor species is allowed to reverse in the bound state.[2] For the side chain, signals from N^{ϵ} and its associated proton both shift ∼0.5 ppm upfield and there is a complete loss of signal from the side chain amino groups. The loss of signals from the side chain amino groups parallels a >80% diminution in the intensity of all ^{15}N and ^{1}H signals from Arg-8 in the bound state (Figure 4). This does not appear to arise from an increase in Arg-8 correlation time, since the line width of the $^{13}C^{\zeta}$ signal was not increased by binding. Moreover, the loss of intensity was manifested only in HMQC spectra and not in direct ^{13}C or ^{1}H spectra. The results are attributed to the effects of chemical exchange processes and indicate a significant restructuring of the environment of Arg-8 when peptide is bound (see Discussion).[3]

A relevant feature of the spectra of the unliganded protein is that there is no evidence of a difference in Arg-8 environment between NP monomers and dimers. At pH 6.7, the unliganded protein is ∼50% dimer by weight in the NMR sample, as can be seen from its 1D proton spectrum (Figure 5). The two peaks, located at 0.27 and 0.20 ppm at this temperature, have been assigned to monomer and dimer, respectively (Peyton et al., 1986). At pH 3.1, the dimerization constant is increased (Peyton et al., 1986) and a single peak representative of the dimer is seen. Nonetheless, there are no signals present in the pH 6.7 $^{1}H\{^{15}N\}$ spectra that are not also present in the pH 3.1 spectra (Tables 3 and 4). Since exchange between monomer and dimer is very slow on the NMR time scale (Pearlmutter, 1979; Breslow et al., 1992), the results point to a lack of effect of dimerization on Arg-8.

Comparison of the pK_a of Glu-47 in Bovine Neurophysins-I and -II in the Unliganded State. Subsequent to the completion of the NMR studies, data were obtained on the crystal structure of a derivative of BNP-II (des 1−6 BNP-II) in the unliganded state, indicating several close van der Waals contacts and potential hydrogen bonding between the two $N^{\eta}H_2$ groups of Arg-8 and the Glu-47 carboxylate and a

[2] Assignments in the bound state are less secure than those in the unbound state, due to the large decrease in signal intensity associated with binding and to the changes in chemical shift in one or both dimensions. Discrimination between cis and trans amide signals is particularly difficult, since (see Figure 4) the amide signals did not differ markedly in intensity in this state, suggesting a change in the relative populations of the two states. None of the conclusions are affected by these uncertainties.

[3] The chemical shift changes of the amide and imino signals are unlikely to represent deshielding/shielding effects of the oxytocin tyrosine ring, which approaches these atoms at distances in the 9.5−12 Å range in the crystal structure of liganded BNP-II.

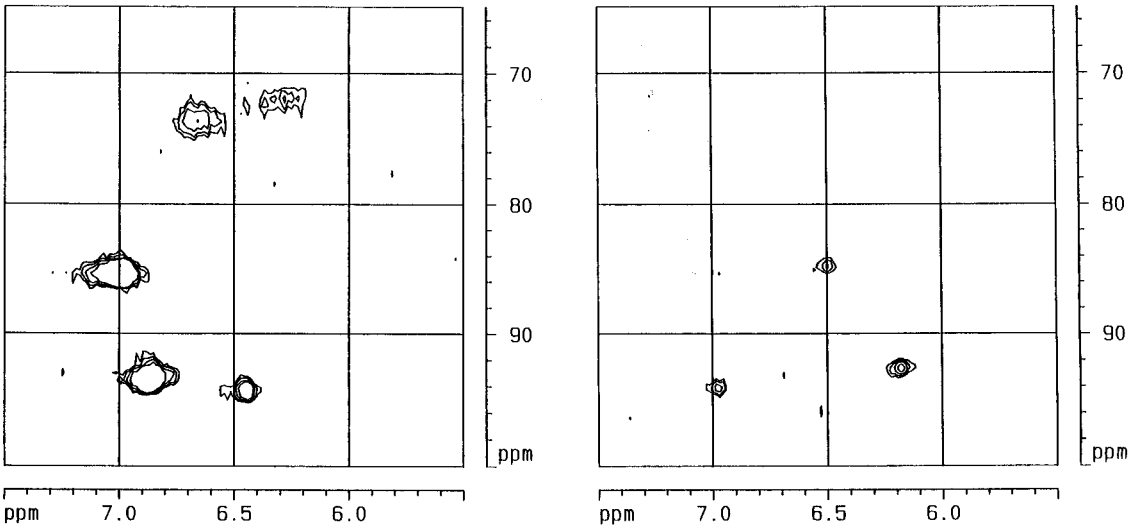

FIGURE 4: $^1H\{^{15}N\}$ HMQC spectra of Boc *des 1−7 BNP-I (~1 mM) at pH 6.7 in the unbound state (left) and at pH 6.1 in the presence of 2 mM oxytocin (right). See Figure 3 legend for other descriptions. The two spectra were processed identically; differences in signal shapes in the two spectra reflect differences in signal intensity.

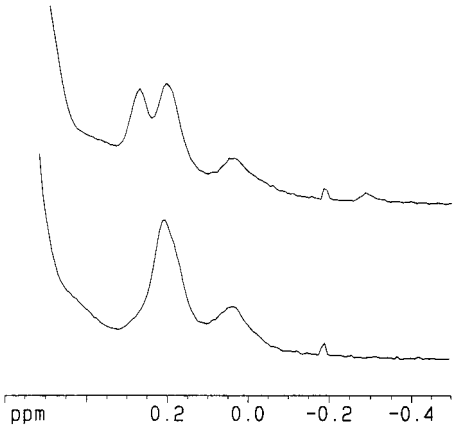

FIGURE 5: 1D proton spectra of Boc *des 1−7 bovine NP-I at pH 6.7 (upper spectrum) and pH 3.1 (lower spectrum) in the upfield aliphatic region. The sample is the same as that shown in Figure 4 prior to the addition of oxytocin (temperature = 5 °C).

strong hydrogen bond from one $N^{\eta}H$ to a backbone carbonyl group (C.-K. Wu, J. P. Rose, C. Zheng, E. Breslow, and B.-C. Wang, manuscript in preparation). This result was at variance with the relatively unperturbed guanidinium NMR spectrum in unliganded BNP-I but offered a possible explanation of the difference between the two proteins in the tryptic susceptibility of the Arg-8 bond in the unliganded state. Accordingly, we set out to determine whether the apparent structural difference between the two proteins was manifested in other properties.

The presence or absence of a salt bridge in the unliganded state should be reflected in the pH dependence of peptide binding, since the protonated α-amino group of the peptide interacts with the carboxylate ion of Glu-47 (Chen et al., 1991). Binding affinity should diminish as the fraction of Glu-47 in the protonated form in the unbound state increases; the presence of a salt bridge in the unliganded state should lower the pK_a of Glu-47. Early binding studies indicated a nonidentical dependence on pH for the binding of oxytocin and of small peptides to BNP-I and -II, with the data suggesting that the pK_a of the responsible carboxyl was ~0.6 pH unit higher in BNP-I than in BNP-II (Camier et al., 1972; Breslow & Gargiulo, 1977). NMR studies (Blumenstein et al., 1979) subsequently provided evidence that the carboxyl involved was the active site carboxyl, now identified as Glu-47. However, analysis of the effects of pH on binding is complicated by effects of pH on dimerization and the higher binding affinity of the dimer. Thus, the dimerization constant of BNP-I (Peyton et al., 1986), but not of BNP-II (Nicolas et al., 1980), increases markedly at low pH. Here, we again probed Glu-47 pK_a values, this time by measuring the decrease in binding affinity for each neurophysin (mononitrated) on lowering the pH from 6.2 to 3.5 and correcting for pH effects on dimerization. The dimerization constant of the two proteins was determined at each pH by NMR (see Methods), and the assumption was made that only binding to dimer was statistically significant (Breslow et al., 1991). Table 5 shows the measured binding and dimerization constants at each pH and the corrected binding constants. Assuming that only binding to the ionized carboxyl group

Table 5: pH Dependence of Peptide (Phe-Tyr-NH_2) Binding, of Dimerization, and of the Optical Activity of Nitrotyr-49 in the Region of Carboxyl Group Titration (Comparison of Bovine NP-I and -II)[a,b]

	BNP-I	BNP-II	des 1−6 BNP-II
dimerization K (M^{-1})			
pH 6.2	1.0×10^4	5.8×10^3	
pH 3.5	6.5×10^5	1.1×10^4	
binding $K_{observed}$ (M^{-1})			
pH 6.2	11400	12000	
pH 3.5	726	1440	
binding K_{dimer} (M^{-1})			
pH 6.2	30000	41380	
pH 3.5	825	3600	
apparent pK_a, Glu-47	5.05	4.52	
apparent pK_a, nitroTyr-49 transition	5.10(±0.05)	4.50(±0.05)	4.45

[a] Conditions: 0.1 M Na phosphate buffer, protein concentration, 5×10^{-5} M, 25 °C. [b] Dimer binding constants were calculated from observed binding constants, assuming that only binding to dimer is statistically significant (see Methods), using the relationship: binding K_{dimer} = binding $K_{observed}$/weight fraction dimer. The apparent pK_a of Glu-47 was calculated from the derived dimer binding constants at pH 6.2 and 3.5 with the relationship: fraction COO^- at pH 3.5 = binding $K_{dimer(pH3.5)}$/binding $K_{dimer(pH\ 6.2)}$. This ignores binding to the protonated carboxyl group which occurs with 8/1000 the affinity of that to the ionized carboxyl (Breslow & Gargiulo, 1977). If this binding is considered, derived pK_a values are 5.19 and 4.57 for BNP-I and -II, respectively.

is significant in this pH range (binding to the protonated carboxyl group occurs with less than 1/100 of the affinity of that to the ionized carboxyl [Breslow & Gargiulo, 1977]), the results give pK_a values for Glu-47 that are 0.5 pH unit higher for BNP-I than for BNP-II. The difference increases to 0.6 pH unit if binding by the protonated carboxyl group is considered (see Legend, Table 5). The results provide additional argument for a difference in the environment of Glu-47 in the two proteins (also see Discussion).

CD studies of mononitrated neurophyin had also previously indicated that the optical activity of nitrotyrosine-49 was strongly pH-dependent in the region of carboxyl titration; the pK_a of of the responsible carboxyl group was ~0.6 pH unit higher in BNP-I than in BNP-II (Breslow & Gargiulo, 1977). Identification of the carboxyl involved as the active site carboxyl was, as above, insecure at that time. Accordingly, we redetermined the pH dependence of the nitrotyrosine CD signal under the same conditions of buffer and protein concentration used for binding studies. The derived pK_a values for each neurophysin (Table 5) are the same, within experimental error, as the pK_a values derived from the binding studies. The results provide strong support for the concept that the the same carboxyl group (Glu-47) is responsible for the pH dependence of binding and the pH dependence of nitrotyrosine optical activity. For comparison with the X-ray crystallography results, the pK_a of the transition in des 1−6 mononitrated BNP-II was also determined and shown to be similar to that in BNP-II (Table 5).

DISCUSSION

The present results provide an internally consistent view of bovine NP-I in the unliganded state in which the Arg-8−Glu-47 salt bridge is weak, the Arg-8 is vulnerable to tryptic attack, and the pK_a of Glu-47 is normal;[4] the presence of a salt bridge in the unliganded state should reduce the tryptic susceptibility of the Arg-8 bond and lower the pK_a of Glu-47. These studies also confirm the difference between the two bovine neurophysins in the pH dependence of binding—and therefore in the pK_a of Glu-47—and indicate that Glu-47 is also responsible for the pH dependence of the nitrotyrosine-49 optical activity. Additionally, an explanation of the difference between the two neurophysins in the tryptic susceptibility of the Arg-8 peptide bond and in the pK_a of Glu-47 is provided by the apparent difference between the two in the extent of salt bridge formation between Arg-8 and Glu-47 in the unliganded state. Close interactions between the Glu-47 carboxyl and the Arg-8 guanidinium group are seen in the crystal strucure of unliganded des 1−6 BNP-II,[5] while such interactions are weak in unliganded BNP-I, as evidenced by NMR and discussed further below.

The conclusion that the NMR data indicate a lack of strong interaction betweeen Arg-8 and Glu-47 in BNP-I rests on comparison of Arg-8 side chain chemical shifts in the protein and in Arg and Boc-Arg derivatives. For the unliganded des 1−7 protein and its Boc derivative at neutral pH, a *maximum* downfield shift in the protein of 0.5 ppm for the N$^{\eta}$ signals is observed relative to small molecule signals (Table 4). This compares with downfield shifts of 2−5 ppm for Arg N$^{\eta}$ signals of the phospholipase Cγ1 SH-2 domain upon salt bridge formation with phosphotyrosine (Yamazki et al., 1995) and a downfield chemical shift of ~3 ppm associated with hydrogen bonding of the NH of the Phe-3 of vasopressin upon its interaction with neurophysin (Live et al., 1987; Chen et al., 1991; Rose et al., 1996). Comparison of the observed proton shifts for the guanidinium amino protons is more complex, since (under these conditions) each observed signal is the average of two rapidly equilibrating signals from the two protons that are attached to the same nitrogen (Yamazaki et al., 1995), each of which might be affected differently by carboxylate interactions. Nonetheless, the maximum downfield shift here is 0.15 ppm, which compares with averaged downfield shifts of 1−2 ppm for each pair of N$^{\eta}$ protons in the salt-bridged SH-2 domain (Yamazaki et al., 1995) and 0.8 ppm for the hydrogen-bonded NH proton of Phe-3 of vasopressin (Live et al., 1987). The small shifts of Arg-8 side chain signals in the unliganded protein relative to Boc-Arg argue most directly for negligible or weak interactions with Glu-47. The possibility that the small shifts represent interactions in an aqueous environment, as opposed to potentially nonaqueous environments in the SH2 domain and vasopressin systems, does not alter the argument, since neither a salt bridge nor inter-residue hydrogen bond in an aqueous environment would be expected to be strong. Nonetheless, that weak interactions of some type are present is supported by the effects of carboxyl group protonation on chemical shift (Table 4) and perhaps also by the reduced solvent exchange rate of the N$^{\eta}$H$_2$ protons in the protein relative to Boc-Arg at neutral pH. Additionally, since the Arg-8 side chain is involved in a salt bridge in the liganded state (see below), the fact that its correlation time is not measurably affected by binding suggests its involvement in some type of interaction in the unliganded state. In any event, interactions in the unliganded state differ from those in the liganded state, as evidenced by the large changes in side chain resonances associated with oxytocin-binding. Similarly, comparison of the crystal structures of liganded (Rose et al., 1996) and unliganded BNP-II derivatives (manuscript in preparation) has recently demonstrated a binding-induced rearrangement of contacts *within* the BNP-II salt bridge.

The present results also demonstrate binding-induced conformational change in the amino-terminal region of Arg-8, which can be attributed to effects of the Boc group. In the unliganded state, the Arg-8 α-amino group shows little evidence of interaction with other elements of the protein; chemical shifts of the cis and trans Arg-8 amide signals in the unliganded Boc des 1−7 protein are almost identical to those in Boc-Arg-Gly-NH$_2$. Since the Boc group can be viewed as a close but bulkier analog of Val-7 of the native protein (Figure 1), the results suggest that Val-7 and the amino terminus of Arg-8 extend away from the protein in the unliganded state. This is not the case in the liganded state. Binding-induced changes occur in the amide N and its associated proton that are significantly different for the cis and trans isomers. Taken together with the lower binding affinity of the Boc derivative than of its unblocked coun-

[4] The calculated pK_a of 5.1 is actually higher than the value of ~4.5 typically assumed for a normal Glu side chain carboxyl group. This potentially reflects the presence of a neighboring Glu at position 46 and/or a low dielectric constant surrounding the Glu-47 carboxyl.

[5] The possibility that the strength of the salt bridge differs in the crystal and in solution cannot be precluded. However, the assumption that the crystal structure is applicable in solution permits a direct and internally consistent explanation of the difference in properties of the two bovine neurophysins.

terpart (Table 2), the results suggest that restructuring is necessary to accommodate the Boc group in the liganded state and argue for a closer packing of Val-7 with other elements of the protein when oxytocin is bound. Consistent with this, the proton NMR signals from the Boc group in the protein, which are similar to those in Boc-Arg in the unliganded state, are markedly broadened by the binding of either oxytocin or small dipeptides to the hormone-binding site (data not shown). Conclusions from the NMR data about the nature of binding-induced conformational changes at residue 7 are now also supported by recent comparisons of the crystal structures of liganded BNP-II (Chen et al., 1991; Rose et al., 1996) and unliganded des 1−6 BNP-II (manuscript in preparation), which indicate the gain of van der Waals contacts between residue 7 and other elements of the protein in the bound state and also to hormone residue 3 when oxytocin is the ligand.

Although the NMR spectra indicate major changes in the environment of Arg-8 when peptide is bound, the absence of signals from the side chain amino groups in the bound state precludes assessment of salt bridge formation per se in this state from the NMR data alone. However, the chemical replacement data argue for a salt bridge in the bound state of BNP-I essentially identical to that seen in crystals of BNP-II complexes, as would also be predicted by the strong homology between the two proteins (Figure 1) and their very similar binding behavior (Breslow & Burman, 1990). In the crystal structure of liganded BNP-II, the terminal amino groups of Arg-8 interact with the Glu-47 carboxylate, which is also bonded to the protonated peptide α-amino group (Chen et al., 1991). Therefore, the negative charge on Glu-47 is shared by two cationic groups, implying that the interactions are not solely electrostatic. The fact that Cit can substitute for Arg-8 with half of its effectiveness argues for equal roles of hydrogen bonding and electrostatics in the interaction and is consistent with this structure. The ability of His to effectively substitute and the lack of effectiveness of the other amino acids examined are also clearly consistent with the crystal structure. Probably the sole surprise is the similar effectiveness of the L- and D-Cit derivatives, but preliminary modeling suggests that the terminal amino groups of both isomers should be able to interact with Glu-47 in the bound state.

The loss of signals from the Arg-8 terminal amino groups in the bound state results at least in part from exchange processes that adversely impact on the HMQC intensity of all the Arg-8 NH signals (see Results). The decrease in intensity most generally suggests that Arg-8 samples different environments in the bound state on a time scale (∼5 ms range) similar to that involved in the HMQC magnetization transfer processes, with equilibration among different states either absent in the unliganded state or, more likely, occurring on a shorter time scale. Exchange between free and bound states is also a potential contributor, but probably occurs on a time scale that is too long (e.g., Pearlmuttter & Dalton, 1980); the separate signals from free and bound states observed at incomplete saturation (Tables 4 and 5) indicate a time scale ≥15 ms. Rotation specifically around bonds in the guanidinium group might be constrained by factors additional to those affecting the rest of the residue. The two hydrogens on each NH_2 are normally in fast chemical exchange at 5 °C due to rotation around the $C^{\zeta}-N^{\eta}$ bond, and a single proton signal is seen from each pair (Yamazaki et al., 1995). Salt bridge formation lowers the rate of chemical exchange because of increased barriers to rotation around the $C^{\zeta}-N^{\eta}$ bond, in the extreme allowing the detection of separate signals from the two protons attached to the same N (Yamazaki et al., 1995). An intermediate rate of exchange, as might pertain here, would lead to signal loss.[6]

The NMR results point to a similar environment for Arg-8 in unliganded monomers and dimers, arguing against a mechanistic role for Arg-8 in effecting the conformational differences between these two states. The importance to allosteric mechanism of specific Arg-8 interactions in the unliganded state is in general brought into question by the apparent differences between BNP-I and -II in these interactions, as opposed to their similar allosteric behavior (e.g., Breslow et al., 1991). Thus, the principal role of Arg-8 does not appear to reside in the constraints it might impose on the conformation of the unliganded state. On the other hand, major changes at and around Arg-8 are induced by ligand binding in both neurophysins. Assuming that interactions in the bound state are the same for both proteins, a view supported by the studies here, the results suggest that effects of Arg-8 on neurophysin behavior derive principally from its influence on the conformation of the liganded state, with interactions of Arg-8 in this state participating to stabilize a conformation that has both an increased dimerization constant and a stronger affinity for peptide.

[6] Detectability of the guanidinium NH_2 signals was not improved by an an increase in temperature to 30 °C, suggesting a role of multiple exchange processes with different temperature sensitivities.

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