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CONTRIBUTION OF THE PEPTIDE BACKBONE TO THE BINDING OF PEPTIDES AND HORMONES TO NEUROPHYSIN*

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SUMMARY

The carboxamide terminus of dipeptide analogs of oxytocin and vasopressin was shown to participate in binding to the principal hormone-binding site of neurophysin. Replacement of the carboxamide group of L-phenylalanyl-L-tyrosine amide by the corresponding ethyl ester, a hydroxymethyl group or a hydrogen led to large decreases in binding affinity associated with less negative enthalpies and entropies of binding. Binding affinities were: amide > ester > hydroxymethyl > hydrogen. It is proposed that the carboxamide group hydrogen-bonds to the protein and that, by analogy, segments of the backbones of oxytocin and vasopressin are hydrogen-bonded to neurophysin in hormone-neurophysin complexes.

Binding of the hormones oxytocin and vasopressin to their carrier protein neurophysin provides a model system for the study of non-covalent protein-protein interactions (1). Interactions at the principal binding site of neurophysin have been studied using the hormones and dipeptide amide and tripeptide amide analogs of the amino-termini of the hormones; the smaller peptides bind with the same qualitative features as the larger molecules (1,2). Based on these studies, the interaction has been defined as principally involving a bond between the protonated α-amino group of the hormones and a carboxyl side-chain on neurophysin and apolar interactions of the side-chains of hormone residues 1-3 with unknown residues on the protein (1,2).

Interaction of both the hormones and small peptide amides with the principal site of neurophysin is enthalpy-driven and accompanied by a negative entropy change (1,3,4), but the origin of the negative enthalpy is unassigned (1). One possible source of negative enthalpy changes in protein-protein interactions is hydrogen-bonding (e.g., 5). Accordingly, we were interested

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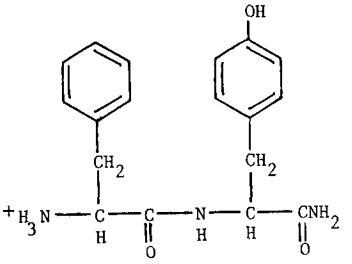
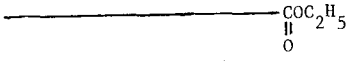
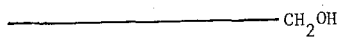
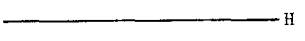
Table I: Thermodynamic Parameters for Peptide-Binding

Structure [a]	$K_{assoc.}$ [b] (M^{-1})	$-\Delta G°$ [b] (Kcal/mol)	$-\Delta H$ (Kcal/mol)	$-\Delta S$ (eu)
	9000	5.4	11.0	18.7
	5400	5.1	9.5	14.9
	2500	4.6	7.4	9.5
	500	3.7	5.1	4.9

[a] Structures from top to bottom are Phe-Tyr$CONH_2$, Phe-Tyr$COOC_2H_5$, Phe-TyrCH_2OH, Phe-TyrH.

[b] Values reflect the apparent association constant at half saturation and 24°C (see text).

in the possibility that the peptide backbone might participate in hydrogen-bonding with neurophysin in the bound state and contribute to the negative enthalpy of binding; backbone contributions to hormone-neurophysin interaction have not previously been invoked. To test this possibility, we synthesized several analogs[1/] of L-phenylalanyl-L-tyrosine amide (Phe-Tyr$CONH_2$)[1/], a dipeptide that binds strongly to the principal binding site of neurophysin (3), and studied their binding to neurophysin. The analogs synthesized, Phe-Tyr-$COOC_2H_5$, Phe-TyrCH_2OH and Phe-TyrH, represent modifications of the carboxamide terminus in which opportunities for hydrogen-bonding at the C-terminus are systematically reduced; structures are shown in Table I. Because the carboxa-

1/ In order to clearly show the structural relationships among the peptides, we have used non-standard peptide abbreviations as follows: Phe-Tyr$CONH_2$, L-phenylalanyl-L-tyrosine amide; Phe-Tyr$COOC_2H_5$, L-phenylalanyl-L-tyrosine ethyl ester; Phe-TyrCH_2OH, L-2-(L-phenylalanylamido)-3-(p-hydroxyphenyl)-1-propanol, alternatively called L-phenylalanyl-L-tyrosinol; Phe-TyrH, L-phenylalanyl-tyramine. Other abbreviations are: CD, circular dichroism.

mide terminus of Phe-TyrCONH$_2$ represents the backbone region of the hormones between residues 2 and 3, effects of these modifications should reflect the role of this region of the hormone backbone.

MATERIALS AND METHODS

Bovine neurophysin-II, nitrated at its single tyrosine, was prepared as previously described (6) and further purified by affinity chromatography (Chang, P. and Breslow, E., manuscript in preparation). L-Phenylalanyl-L-tyrosine amide, L-phenylalanyl-L-tyrosine and CBZ-L-phenylalanine were from Vega Biochemicals and tyramine was from Mann Research Laboratories. All general chemicals were reagent grade and water was deionized. Peptide and amino acid purity were checked by TLC on Whatman LK5DF plates and molar extinction coefficients were obtained by quantitative amino acid analysis. All commercial peptides and the final products of synthesis (see below) were homogeneous on TLC as monitored by UV, ninhydrin and I_2 vapor.

Phe-TyrCOOC$_2$H$_5$ was prepared by refluxing L-phenylalanyl-L-tyrosine and ethanol with thionyl chloride. Amino acid analysis of the purified product gave equimolar quantities of phenylalanine and tyrosine with an extinction coefficient of 1340 $M^{-1}cm^{-1}$ at 275 nm in 0.1 N HCl. Phe-TyrCH$_2$OH was synthesized by refluxing CBZ-Phe-TyrCOOC$_2$H$_5$ with LiAlH$_4$ in ethyl ether for 30 min followed by deblocking by catalytic hydrogenation; as evidenced by IR, the internal amide bond was not reduced, but the C-terminal carboxyl was no longer present. Phe-TyrH was prepared by carbodiimide coupling of CBZ-phenylalanine and tyramine followed by deblocking by catalytic hydrogenation. Quantitative amino acid analysis on Phe-TyrCH$_2$OH and Phe-TyrH indicated only the presence of phenylalanine, with both the alcohol derivative of tyrosine (2-amino-3-[p-hydroxyphenyl]-1-propanol) and tyramine retained on the analyzer column. Per mole of phenylalanine, both peptides had UV absorption spectra virtually identical to that of Phe-TyrCONH$_2$ with maxima in 0.1 N HCl at 275 nm and extinctions at this wavelength of 1350 $M^{-1}cm^{-1}$ for Phe-TyrCH$_2$OH and 1500 $M^{-1}cm^{-1}$ for Phe-TyrH.

CD binding studies were performed as previously described (7), monitoring the effect of peptides on the CD spectrum of the protein nitrotyrosine. In the present studies a Jobin-Yvon Mark 5 Dichrograph was used and the temperature maintained by a water bath circulating to an aluminum cuvette holder. All studies were conducted using an initial protein concentration of 2mg/ml in 0.16 M KCl, 0.005 M citrate, pH 6.2 in a 1 cm cuvette; volume changes over the course of each study were less than 8%. The CD spectrum of the saturated protein was obtained by addition of solid Phe-TyrCONH$_2$ regardless of the peptide used to partially saturate the protein (7); for the modified peptides, as for all peptides studied so far, the change in nitrotyrosine CD has been found to depend only on the fractional occupancy of the hormone-binding site and to be independent of the identity of the bound peptide (7). Calculations of the moles of peptide bound per mole of neurophysin polypeptide chain ($\bar{\nu}$) and the concentration of free peptide were as previously described (3,7).

RESULTS

Fig. 1 shows Scatchard plots, obtained under identical conditions, of the binding of Phe-TyrCONH$_2$ and its derivative peptides at 24°C. The Phe-TyrCONH$_2$ data are identical to those obtained in an earlier more comprehensive study of

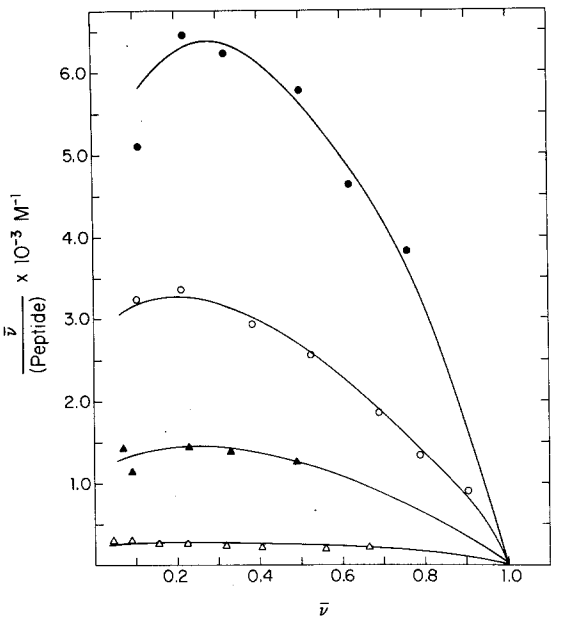


Figure 1. Scatchard plots of the binding of Phe-TyrCONH$_2$ and its derivatives to nitrated bovine neurophysin-II. ● , Phe-TyrCONH$_2$; ○ , Phe-Tyr-COOC$_2$H$_5$; ▲ , Phe-TyrCH$_2$OH; △ , Phe-TyrH. Conditions: Temp = 24°C, pH 6.2. Each isotherm represents a single binding study. Peptide insolubility limited the highest values of $\bar{\nu}$ that could be attained with Phe-Tyr-CH$_2$OH and Phe-TyrH. For Phe-TyrCH$_2$OH, the highest value of $\bar{\nu}$ typically attained was ∿0.75 while that for Phe-TyrH was ∿0.65.

this peptide under the same conditions (3). The results indicate a large essentially systematic effect of carboxamide modification on binding. All derivative peptides show reduced affinity relative to Phe-TyrCONH$_2$; the most weakly binding peptide, Phe-TyrH, has approximately 1/20 the affinity of Phe-TyrCONH$_2$. Within experimental error, all peptides show weak binding cooperativity, although it is not certain that the magnitude of the cooperativity is identical in all cases. This cooperativity has been shown to arise from ligand-facilitated protein dimerization (2) and weak positively cooperative interactions between the two strong binding sites of the dimer (2,3). Accordingly, to simplify comparison of the different binding isotherms, binding constants (Table I) are calculated from data at $\bar{\nu}$ = 0.5 (3) using the simple relationship:

$$K_{assoc.} = \frac{\bar{\nu}}{(1-\bar{\nu})\ \ (\text{Free peptide})} \qquad (1)$$

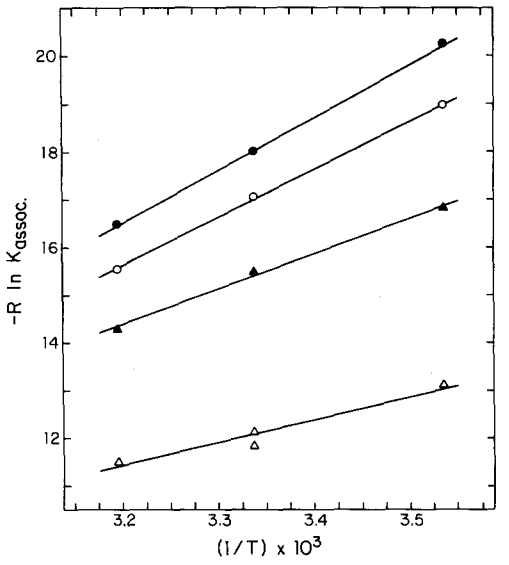


Figure 2. Van't Hoff plots of the effect of temperature on the binding of Phe-TyrCONH$_2$ and its derivatives to nitrated bovine neurophysin-II. Temperature is given in degrees K as recorded in the CD cuvette. Values of $K_{assoc.}$ were calculated as described in the text. ● , Phe-TyrCONH$_2$; ○ , Phe-TyrCOOC$_2$H$_5$; ▲ , Phe-TyrCH$_2$OH; △ , Phe-TyrH.

The van't Hoff enthalpy of binding of each of the peptides was determined from the temperature-dependence of the binding affinity. Van't Hoff plots, representing data at 10°C, 24°C and 40°C are shown in Fig. 2 and were essentially linear over the temperature range studied. The results show that the decreases in binding affinity associated with carboxamide modification are accompanied by large enthalpy changes. This is seen most clearly in Table I where values for $\Delta G°$, ΔH and ΔS for each peptide are listed. Decreases in binding affinity are seen to reflect decreases in the absolute value of the negative enthalpy change, partially compensated for by a less negative ΔS of binding. Note that values of ΔH and ΔS calculated for each peptide are independent, within experimental error, of the value of $\bar{\nu}$ from which $K_{assoc.}$ is calculated, provided the same value of $\bar{\nu}$ is used for all studies. It is also relevant that the van't Hoff enthalpy contains small contributions from changes in nitrotyrosine-49 ionization associated with binding and from changes

in peptide α-amino ionization with temperature (3). These are not corrected for in the present analysis but should be essentially identical for all the peptides.

DISCUSSION

The decreased binding affinities of the modified peptides relative to Phe-TyrCONH$_2$ can in principle arise either from (a) steric hindrance at the binding site, (b) conformations of the modified peptides in the free state that are less favorable to binding than that of Phe-TyrCONH$_2$ and/or (c) a decreased number of bonding interactions between the peptides and protein in the complex. Steric hindrance is probably not a significant factor because the modified peptides are, in most respects, smaller than the parent peptide. One exception is the ethyl ester, but previous experience (7,8) suggests that the $-OC_2H_5$ substitution on the carboxyl should not present a steric problem. Additionally, trends among the peptides in the ΔH and ΔS of binding suggest that conformational factors are not the principal determinants of binding differences. Thus, a likely origin of conformational effects would be an increased flexibility of the smaller modified peptides than of the parent peptide in the unliganded state such that the relative loss of motional freedom on binding was greater for the modified peptides. However, the data in Table I indicate that the greater the decrease in binding affinity, the less the entropy loss associated with binding.

Alternatively, the trends in ΔG°, ΔH and ΔS are consistent with the postulate that the decreases in affinity associated with modification result from a decrease in hydrogen-bonding interactions between peptide and protein. Hydrogen-bonding is considered to be an enthalpy-driven reaction associated with a negative entropy change (e.g., 5,9) and each of the modifications leads to a less negative enthalpy and a less negative entropy of binding (Table I). Moreover, there is a semi-systematic relationship between the number of hydrogen bonds available to each peptide and the thermodynamic parameters. Thus,

if we assume that two hydrogen bonds at the $-CONH_2$ terminus are possible for Phe-Tyr$CONH_2$ (one at the -C=O and the other involving the -NH), none for Phe-TyrH, and one each for the ester (the -C=O) and the alcohol (the -OH possibly substituting for the amide -NH), this correlates with the fact that the thermodynamic parameters for the ester and alcohol fall between those of the parent amide and Phe-TyrH. Within the assumption that differences among the peptides principally reflect differences in hydrogen-bonding, an estimate can be made of the strength of these hydrogen bonds. In particular, the different thermodynamic parameters of Phe-Tyr$CONH_2$ and Phe-TyrH, which differ by two hydrogen bonds, indicate that, for the formation of a single hydrogen bond, $\Delta G^\circ = -0.9$ Kcal/mole, $\Delta H^\circ \overset{\sim}{=} -2.9$ Kcal/mole and $\Delta S = -6.9$ eu. Additionally, data for Phe-Tyr$COOC_2H_5$ and Phe-TyrCH_2OH suggest that the two hydrogen bonds at the carboxamide terminus are not thermodynamically equivalent here, loss of the carbonyl oxygen leading to a larger change in thermodynamic parameters than loss of the -NH (Table I); however, apolar interactions of the $-C_2H_5$ group may contribute to the relatively strong affinity of the ester. In either event, the possibility that hydrogen-bonding contributes significantly to the strength of neurophysin-peptide complexes is at variance with the view based on the model studies (9,10) that hydrogen-bonding cannot contribute a significant net stability to the structures of proteins in solution.

In sum, our results demonstrate that the carboxamide terminus of dipeptide amides plays an important role in the stability and enthalpy of neurophysin-dipeptide complexes. We propose that this stabilization arises from hydrogen-bonding and that, by analogy, the peptide backbone between residues 2 and 3 of oxytocin and vasopressin is hydrogen-bonded to neurophysin in the bound state. Hydrogen-bonding of other regions of the hormone-backbone, particularly between residues 1 and 2, may also occur and may be a factor in the large changes in hormone α-carbon NMR chemical shifts associated (11) with binding to neurophysin.

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