Enthalpies of Ligand Binding to Bovine Neurophysins†

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Received July 6, 1984

ABSTRACT: Flow microcalorimetry and batch microcalorimetry have been used to survey the energetics of ligand binding by bovine neurophysins I and II. Calorimetry studies were supplemented by van't Hoff analyses of binding constants determined by circular dichroism. Free energies of binding of a series of di- and tripeptides that bind to the strong hormone binding site of neurophysin were partitioned into their enthalpic and entropic components. The results indicate that, at 25 °C, the binding of most peptides is an enthalpy-driven reaction associated with negative entropy and heat capacity changes. Studies elsewhere, supported by evidence here, indicate that the principal component of the negative enthalpy change does not arise from the increase in neurophysin dimerization associated with peptide binding. Accordingly, the negative enthalpy change is attributed to direct bonding interactions with peptide and possibly also to peptide-induced changes in tertiary or quaternary organization. Comparison of the binding enthalpies of different peptides indicated two types of bonding interactions that contribute to the negative enthalpy change of peptide ligation. Substitution of an aromatic- or sulfur-containing side chain for an aliphatic side chain in position 1 of bound peptides led to increases in negative enthalpy of from 1 to 6 kcal/mol, demonstrating that interactions typically classified as hydrophobic can have a significant exothermic component at 25 °C. Similarly, loss of hydrogen bonding potential in the peptide decreased the enthalpy change upon binding, in keeping with the expected enthalpic contribution of hydrogen bonds. In particular, the data suggested that the peptide backbone between residues 2 and 3 and the phenolic hydroxyl group in position 2 participate in hydrogen bonding. Studies of the buffer dependence of the enthalpy changes associated with peptide binding were also carried out. These suggested that binding enthalpies might be influenced by unexpected interactions of neurophysins with certain buffers and revealed significant behavioral differences between neurophysins I and II.

The neurophysins are small, stable, disulfide-rich proteins whose synthesis in the hypothalamus and transport to the posterior pituitary are tightly coupled to synthesis and transport of the peptide hormones oxytocin and vasopressin. [For recent reviews, see Breslow (1979, 1984), Cohen et al. (1979), and Chaiken et al. (1983).] Since large amounts of relatively homogeneous, stable protein can be readily obtained (Hollenberg & Hope, 1968; Breslow et al., 1971), they are accessible to physical analysis. The neurophysins are of particular interest from a thermodynamic point of view because they provide a useful model system with which to analyze the energetics of individual protein–peptide bonding interactions (Breslow, 1975, 1984) and because they exhibit the phenomenon of quaternary enhancement (Mills & Ackers, 1979); self-association to form dimers is promoted by the binding of ligands and vice versa (Nicolas et al., 1976, 1978a,b; Angal & Chaiken, 1982). The structural correlate of the linkage between self-association and peptide binding is a substantial change in the tertiary or quaternary structure of the dimer which occurs upon ligand binding (Rholam et al., 1982; Virmani-Sardana & Breslow, 1983).

In order to arrive at a thorough understanding of peptide binding and of the linkage between binding and self-association in this system, it is necessary to examine the energetics of both processes in detail over a wide range of conditions. Free energies of binding a series of di- and tripeptide analogues of the hormones to mononitrated neurophysin II at pH 6.2 have been determined by Breslow et al. (1973) and used to develop a model of the hormone binding site (Breslow, 1975, 1984). van't Hoff enthalpies of binding selected peptides have also been determined (Breslow & Gargiulo, 1977; Carlson & Breslow, 1981a). Nicolas examined the thermodynamics of oxytocin binding (Nicolas et al., 1976, 1978a) and derived a set of values for the microscopic constants for ligand binding and self-association (Nicolas et al., 1978b). Linkage theory and van't Hoff analysis have also been used to define the thermodynamics of the dimerization reaction over a range of pHs and temperatures (Nicolas et al., 1980).

The initial model of the binding site proposed by Breslow et al. (1973) involved only hydrophobic and electrostatic interactions, leading to the expectation that the enthalpy of binding hormones or hormone analogues would be positive. Early values for the van't Hoff enthalpy for binding L-phenylalanyl-L-tyrosine amide (Phe-Tyr-NH_2),[1] however, were

†Supported by Grant PCM-790643 from the National Science Foundation to N.M.A. and Grants GM-17528 and 5P41GM-27471 from the National Institutes of Health to E.B. The calorimetric studies and new protein purification methods reported here are taken from a thesis submitted to Wesleyan University by B.A.W. in partial fulfillment of the degree of Doctor of Philosophy.
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[1] Abbreviations: CD, circular dichroism; Phe-Tyr-NH_2, L-phenylalanyl-L-tyrosine amide; Met-Tyr-NH_2, L-methionyl-L-tyrosine amide; Met-Tyr-Phe-NH_2, L-methionyl-L-tyrosyl-L-phenylalanine amide; Phe-Phe-NH_2, L-phenylalanyl-L-phenylalanine amide; Ala-Tyr-NH_2, L-alanyl-L-tyrosine amide; $(Cys\text{-}Tyr\text{-}NH_2)_2$, L-cystinylbis(L-tyrosine) amide; Gly-Tyr-NH_2, glycyl-L-tyrosine amide; Leu-Tyr-NH_2, L-leucyl-L-tyrosine amide; Ala-Tyr-Phe-NH_2, L-alanyl-L-tyrosyl-L-phenylalanine amide; Phe-TyrH, L-phenylalanyl-L-tyramine (representing Phe-Tyr-NH_2 in which the terminal carboxamide has been replaced by H); Phe-PheH, L-phenylalanylphenylethylamine (representing Phe-Phe-NH_2 in which the terminal carboxamide has been replaced by H); NP, neurophysin; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; PAGE, polyacrylamide gel electrophoresis; HPLC, high-pressure liquid chromatography; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid.

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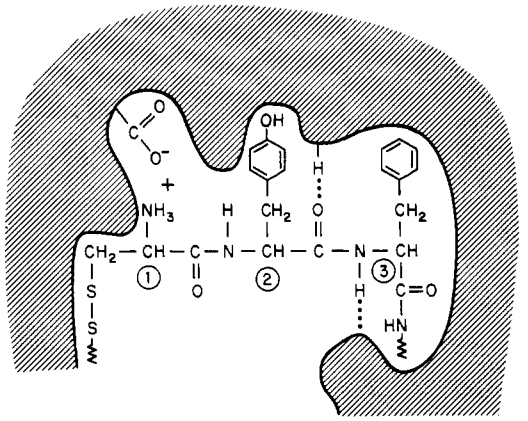

FIGURE 1: Model of the interactions of vasopressin with the strong hormone binding site of neurophysin. Numbers define the hormone residue; the shaded region is the protein. The protein interacts principally with residues 1–3 of the hormone. Interactions involve formation of a salt bridge by the hormone α-amino-group and apolar bonding of the side chains of residues 1–3 [e.g., see Breslow (1979)]; hydrogen bonding of the peptide backbone has also recently been demonstrated (Carlson & Breslow, 1981a). A more complete bonding description is given under Discussion.

found to be approximately −8 kcal/mol at pH 6.2 (Breslow & Gargiulo, 1977), and a still more negative van't Hoff enthalpy was calculated for binding of vasopressin to neurophysin I (Nicolas et al., 1980). Although evidence has recently been obtained that hydrogen bonding interactions between peptide and protein contribute to the negative van't Hoff enthalpies (Carlson & Breslow, 1981a), it was nonetheless of interest to investigate the enthalpy of binding directly by calorimetry and to look for other sources of the negative enthalpy. We have therefore determined calorimetric enthalpies of binding a series of di- and tripeptides to neurophysin II and have supplemented these studies by van't Hoff analyses of peptide binding to nitrated neurophysin II. We have also attempted to examine the linkage between binding of protons and binding of Met-Tyr-Phe-NH_2 and Phe-Phe-NH_2 by surveying the pH dependence of $\Delta H_{binding}$ and by determining apparent enthalpies of binding in a series of buffers with different enthalpies of ionization. Finally, we have evaluated the changes in heat capacity associated with binding of selected peptides to neurophysins I and II.

Figure 1 represents demonstrated interactions between peptides and the hormone binding site of neurophysin. The choice of peptides to be studied here has been governed by this model which, in turn, is derived from a series of physical–chemical studies [see Breslow (1979) for a review] including the determination of peptide binding constants to nitrated neurophysin. In our analyses of the results, we have used the assumption that peptide binding constants determined by using nitrated neurophysin are valid for the native protein. This assumption is supported by the fact that, for all cases where binding constants for the same peptide to both native and nitrated neurophysin have been obtained, the two values are within experimental error [e.g., see Sur et al. (1979)].

Materials and Methods

Calorimetric Studies. Neurophysins I and II were prepared from freeze-dried pituitaries obtained from Pel-Freez (Rogers, AR). The procedures used to prepare crude neurophysins for calorimetric studies were those of Hollenberg & Hope (1968) and Breslow et al. (1971), scaled up and modified so that gram quantities of protein could be obtained with minimal contamination by non-protein UV-absorbing material. Following Rausch et al. (1969) and Audhya & Walter (1977), with minor modifications, crude neurophysins were separated on DEAE-Sephadex (4 × 30 cm) equilibrated with 60 mM Tris-HCl, pH 7.9, with a linear (0.0–0.4 M) NaCl gradient. The flow rate was approximately 12 mL/h, and the rate of change of the gradient was 0.5 M/L. Under these conditions, neurophysins II and I elute at 0.18 and 0.28 M NaCl, respectively, with one of the minor neurophysin components eluting at 0.21 M; the individual peaks were well resolved. Pooled fractions were dialyzed extensively against 0.1 M HCOOH and lyophilized. This procedure eliminates ultraviolet-absorbing material which may account for as much as 25% of the absorbance at 260 nm of protein eluted with the more standard pyridine-containing buffers, improves resolution of neurophysin II from minor neurophysins, and increases yields of neurophysin I. Both neurophysins I and II isolated by this procedure appeared homogeneous by PAGE under the conditions of Breslow et al. (1971), by analytical gel chromatography (Whittaker & Allewell, 1984), and by N-terminal analysis according to the procedures of Gros & Labouesse (1961), Gray (1972), and Niederwieser (1972). Protein concentrations calculated with molar extinction coefficients at 260 nm of 3950 (NP I) and 3580 (NP II) (Carlson & Breslow, 1981b) agreed to within a few percent with concentrations calculated from the dry weight. Typical 260/280-nm absorbance ratios were found to be 1.26 (NP I) and 1.28 (NP II). In order to verify that calorimetric measurements made with different protein samples were truly comparable, ΔH_{app} for binding Phe-Phe-NH_2 to NP II at pH 6.2 was determined for each preparation. In all cases, the deviation from the average value was ≤0.5 kcal/mol.

While both neurophysins I and II appeared homogeneous by the criteria listed above, affinity chromatography on Met-Tyr-Phe-ω-(aminohexyl)agarose (Angal & Chaiken, 1982) in 0.4 M NH_4OAc, pH 5.7, of a sample of neurophysin II, prepared as above and stored at −10 °C as a lyophilized powder for 6 months, indicated that ~23% of the absorbance at 280 nm was associated with material which was not retained by the column. The unbound material was analyzed further by HPLC with a cyanopropyl column and an acetonitrile gradient in triethylammonium phosphate buffer (Chaiken & Hough, 1980). More than 90% of the unbound material eluted with either neurophysin I or neurophysin II, with only a small amount eluting after neurophysin II. The material eluting with neurophysin I may correspond to degraded neurophysin II (I. M. Chaiken, personal communication). Although only one sample was examined, the reproducibility of the calorimetric data (see above) suggested that the amount of inactive material did not vary significantly between preparations. In addition, a number of samples of neurophysin prepared according to the method of Breslow et al. (1971) and subsequently purified on Sephadex G-50 to remove UV-absorbing contaminants have been subjected to affinity chromatography according to the procedure of Rabbani et al. (1982). These studies indicate that protein not bound by the affinity column has a markedly higher UV absorbance than native neurophysin, leading to an approximately 100% overestimate of inactive protein if only UV absorbance is considered. The latter studies suggest that, on a weight basis, the amount of inactive protein in all these preparations is typically 10–15%. In principle, therefore, all of the calorimetric values reported here should be adjusted upward by 10–15%, but this small correction has not been made because it is still regarded as uncertain. Note, however, that such a correction is not necessary for the reported van't Hoff data for the nitrated protein, in part because the nitrated

Table I: Calorimetric Thermodynamic Parameters for Binding Di- and Tripeptides to Neurophysin II at pH 6.2, 25 °C

peptide	concn (mM)	$\Delta G°$ (kcal/mol)[a]	ΔH (kcal/mol)[b] observed	ΔH (kcal/mol)[b] corrected	$\Delta S°$ (cal K^{-1} mol^{-1})[a]
Phe-Tyr-NH_2	1.26	−5.6	−13.2 ± 1.4	−14.0 ± 1.5	−28.2
Met-Tyr-NH_2	3.68	−4.4	−8.2 ± 0.9	−9.4 ± 1.1	−16.8
Met-Tyr-Phe-NH_2	1.20	−5.2	−7.2 ± 0.3	−8.1 ± 0.3	−9.7
Phe-Phe-NH_2	1.20	−5.6	−7.2 ± 0.2	−7.7 ± 0.2	−7.0
Ala-Tyr-NH_2	20	−3.4[c]	−6.1 ± 0.3	−7.0 ± 0.4	−12.1
(Cys-Tyr-NH_2)$_2$	0.75	−5.2[d]	−10.9 ± 1.9	−14.6 ± 2.7	−31.5
Gly-Tyr-NH_2	2.78	−2.3	−5.3 ± 0.2	−9.1 ± 0.3	−22.8
Leu-Tyr-NH_2	4.5	−4.1	−4.7 ± 0.3	−5.7 ± 0.4	−5.4
Ala-Tyr-Phe-NH_2	4.5	−4.1	−4.5 ± 0.3	−5.5 ± 0.4	−4.7

[a] Free energy values given for binding of protonated peptides to nitrated neurophysin II at pH 6.2 by Breslow (1975). These can be converted to unitary free energy values by addition of −2.4 kcal/mol. Similarly, values of ΔS can be converted to unitary entropy values by addition of 8.05 cal K^{-1} mol^{-1}. [b] "Observed" values are those determined at the peptide concentration used for the calorimetric study. "Corrected" values are the observed values corrected to complete saturation with peptide from the binding constants represented by $\Delta G°$. [c] Revised from the value of −3.2 kcal/mol reported by Breslow (1975) to agree with more recent data (e.g., Figure 2). [d] As noted by Breslow (1975), the significance of this value is subject to question, since this ligand is bifunctional and it is not known whether both sites can bind simultaneously. Values reported assume that the ligand binds to only a single neurophysin site.

protein was purified by affinity chromatography (see below).

The amounts of protein required for calorimetry made it necessary to develop a new procedure for recycling the protein. Solutions of protein and peptide were first lyophilized to dryness. Addition of ~3 mL of 1 N HCOOH/150 mg of powder was then sufficient to dissolve most of the peptide, leaving most of the protein and salt as a precipitate which could be spun down with a table-top centrifuge. The precipitate was dissolved in ~200 mL of 0.1 N HCOOH, dialyzed against 3 × 2 L of 0.1 N HCOOH using Spectrapor tubing with a molecular weight cutoff of 3500 (Spectrum Medical Industries, Los Angeles, CA), and lyophilized to dryness.

Calorimetric measurements were made with either a modified (Knier & Allewell, 1978) LKB 10700-1 flow microcalorimeter submerged in a Tronac CTB-1005 waterbath thermostated with a Tronac PTC-40 precision temperature controller to 25.0 ± 0.1 °C or an LKB 2107-010 batch microcalorimeter. The procedures used in the flow experiments have been described by Burz & Allewell (1982); procedures for the batch experiments were those of McCarthy & Allewell (1983). Corrections were made when necessary for heats of dilution of the protein. Protein was equilibrated with the appropriate buffer by overnight dialysis. Any insoluble material was removed by centrifugation. As a result of the experimental design, both protein and ligand solutions are diluted by a factor of 2 in the course of the experiments. Final concentrations of protein were ~1 mg/mL for the flow experiments and ~2 mg/mL for the batch experiments. Final concentrations of ligand were 5–15× published K_d values (Breslow, 1975).

All peptides were purchased from Vega Biochemicals (Tucson, AZ). When not guaranteed by Vega, their purity was verified by thin-layer chromatography. Concentrations determined by weight were verified spectroscopically.

Determination of van't Hoff Binding Enthalpies. Bovine neurophysin II was prepared and mononitrated at its single tyrosine as previously described (Breslow et al., 1973). The nitrated protein was then subjected to affinity chromatography on a column of L-cysteinyl-L-tyrosine amide coupled by disulfide linkage to an agarose support as previously described (Rabbani et al., 1982). Protein retained by the column was chromatographed on Sephadex G-50 in 0.1 N acetic acid to remove the covalent neurophysin dimer formed as a byproduct of the nitration reaction (Breslow et al., 1983) and lyophilized. Repeated affinity chromatography of this material (V. Sardana and E. Breslow, unpublished results) indicated that the dialysis and lyophilization procedures do not lead to any significant inactivation of the initially isolated active protein fractions. The binding of peptides to the active nitrated protein was studied by CD as previously described (Breslow et al., 1973; Breslow & Gargiulo, 1977; Carlson & Breslow, 1981a) with the modification that protein concentrations were additionally verified by quantitative amino acid analysis [e.g., see Carlson & Breslow (1981b)]. Data were analyzed by Scatchard plots as described in the above references. A Jobin-Yvon Mark 5 spectrometer was used for CD studies. This spectrometer is computerized, allowing binding constants to be determined both by following CD changes in the 350-nm nitrotyrosine band at a single wavelength (Breslow et al., 1973) and by integrating changes over all wavelengths in this region. Identical binding constants were obtained regardless of the method used for calculations. Reported binding constants are those calculated from Scatchard plot slopes at half-saturation with peptide (Carlson & Breslow, 1981a).

Peptides used for CD studies are those previously described (Carlson & Breslow, 1981a) and Ala-Tyr-NH_2 and Phe-PheH. Ala-Tyr-NH_2 was obtained from Vega Biochemicals (see above), while Phe-PheH was synthesized as described previously for Phe-TyrH (Carlson & Breslow, 1981a), substituting phenylethylamine for tyramine in the synthesis. Binding studies were conducted at a protein concentration of 2 mg/mL in 0.16 M KCl containing 0.005 M citrate.

Results

Binding Enthalpies of Different Peptides to Native and Nitrated Neurophysins. Apparent enthalpies of binding to unmodified neurophysin II of a series of di- and tripeptide analogues of the hormones were first surveyed by flow microcalorimetry in 20 mM sodium citrate and 0.16 M KCl, pH 6.2 (Table I). All results shown except those for Gly-Tyr-NH_2 are averages of at least three measurements. Under the conditions used, contributions from changes in the state of ionization of the buffer to net heat changes are expected to be negligible, since both the enthalpy of ionization of the buffer and changes in proton equilibria upon complex formation (Breslow et al., 1971) are small. The peptides selected were ones for which free energies of binding to nitrated neurophysin II had been determined under the same conditions (Breslow, 1975), which bound reasonably tightly, and which were commercially available. Peptide concentrations were typically at least $5K_d$, except in the case of Gly-Tyr-NH_2. The concentration of Gly-Tyr-NH_2 that could be achieved was limited by its solubility and the amount of material available to 1.4 K_d. In either case, values of ΔH listed in Table I for this and other peptides are given both as the observed values and as

Table II: Thermodynamic Data at 25 °C for Binding of Peptides to Nitrated Neurophysin II at pH 6.2 As Calculated from van't Hoff Relationships[a]

peptide	$\Delta G°$ (kcal/mol)	$\Delta H°$ (kcal/mol)	ΔC_p (cal K^{-1} mol^{-1})	$\Delta S°$ (cal K^{-1} mol^{-1})
Phe-Tyr-NH_2	−5.6	−9.6 ± 0.5[b]	−410	−13.4
Phe-Phe-NH_2	−5.6	−7.3 ± 0.9[b]	−430	−5.7
Ala-Tyr-NH_2	−3.4	−5.7	−406	−7.4
Phe-TyrH	−3.6	−6.2	−350	−8.7
Phe-PheH	−3.7	−6.0	−520	−7.7

[a] For all peptides, values of ΔH were calculated by the van't Hoff equation from values of $\Delta G°$ at 283, 297, and 313 K by using the approximation that ΔH calculated between adjacent temperatures represented ΔH at the interval midpoint. The difference in ΔH calculated between 283 and 297 K and between 297 and 313 K was then used to calculate ΔC_p by using the relationship $\Delta H_T = \Delta H_{0K} + \Delta C_p(T)$ where ΔH_T is the observed ΔH at any temperature and ΔH_{0K} is the value of ΔH at absolute zero. This relationship was then used to calculate the value of $\Delta H°$ at 298 K (25 °C). For several peptides, such as Phe-Tyr-NH_2, values of $\Delta G°$ were also available from more closely spaced temperature intervals (Figure 2). Values of ΔH and ΔC_p obtained by using all the temperature data for these peptides deviated by less than 10% from values obtained by using only 283, 297, and 313 K. [b] Deviations from average values were calculated for these peptides from the experimentally observed uncertainty in $\Delta G°$ at each temperature.

the expected values at saturation. Protein concentrations were ~1 mg/mL throughout. At this concentration, the protein will be present as both monomer and dimer since the equilibrium constant for dimerization is on the order of (5–8.5) × 10^3 M^{-1} (Breslow et al., 1971; Nicolas et al., 1980). In the presence of ligand, the protein will exist primarily as dimer [e.g., see Rholam et al. (1982)].

The results given in Table I confirm the results of the earlier van't Hoff analysis for Phe-Tyr-NH_2 (Breslow & Garguiulo, 1977) in that the enthalpies of binding are negative and relatively large. Also shown in Table I are the free energy changes for binding, calculated from the values given by Breslow (1975). It will be noted that the variation in the enthalpies of binding is substantially greater than that in the free energies, indicating that the system shows considerable entropy–enthalpy compensation [e.g., see Lumry & Rajender (1970) and Eftink et al. (1983)]. The differences between Phe-Phe-NH_2 and Phe-Tyr-NH_2, which have been thought to bind very similarly, and Met-Tyr-NH_2 and Leu-Tyr-NH_2, which would be expected to bind very similarly, are particularly striking.

In parallel studies, the van't Hoff binding enthalpies of several peptides to nitrated neurophysin II were determined. The peptides studied were three for which calorimetric data were also obtained (Phe-Tyr-NH_2, Phe-Phe-NH_2, and Ala-Tyr-NH_2) and Phe-TyrH and Phe-PheH (which were not available in sufficient quantities for calorimetric analysis); the latter two peptides are derivatives of Phe-Tyr-NH_2 and Phe-Phe-NH_2, respectively, in which the terminal $-CONH_2$ is replaced by H. Some van't Hoff data for Phe-Tyr-NH_2 and Phe-TyrH have been previously reported (Breslow & Gargiulo, 1977; Carlson & Breslow, 1981a) but were redetermined in the present study to obtain data at more closely spaced temperature intervals and to allow direct comparison with Phe-Phe-NH_2 and Phe-PheH. Binding data as a function of temperature are presented in Figure 2 and apparent enthalpies at 25 °C, as determined from these data, are given in Table II. Data are not corrected for the effects of temperature on the pK_a of the peptide α-amino and protein nitrotyrosine residues. The nitrotyrosine pK_a effect can be shown to provide a relatively small positive enthalpic contribution that is constant for all peptides,[2] and the influence of α-amino protonation

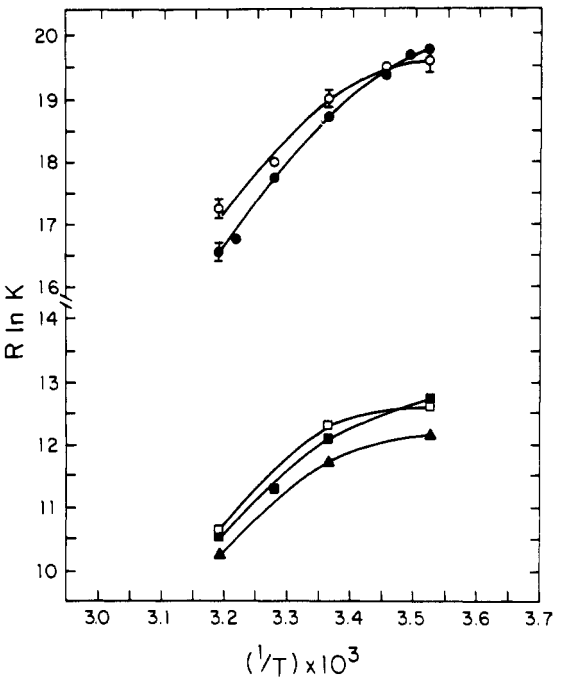

FIGURE 2: van't Hoff plots of the binding of peptides to nitrated neurophysin II at pH 6.2. Binding constants are those calculated at 50% saturation with peptide as described previously (Breslow & Gargiulo, 1977; Carlson & Breslow, 1981a). (●) Phe-Tyr-NH_2; (○) Phe-Phe-NH_2; (■) Phe-TyrH; (□) Phe-PheH; (▲) Ala-Tyr-NH_2.

is discussed in detail subsequently. Nonetheless, when a correction for temperature effects on α-amino protonation is made, as described below, the ΔH value for Phe-Tyr-NH_2 reported here is reduced to −8.4 kcal/mol in excellent agreement with the similarly corrected value of −8.1 kcal/mol reported earlier (Breslow & Gargiulo, 1977). Additionally, comparative $\Delta G°$ values for Phe-Tyr-NH_2 and Phe-TyrH (the latter representing substitution of the terminal carboxamide of Phe-Tyr-NH_2 by a proton) indicate that the same decrease in binding affinity accompanies loss of the terminal carboxamide as reported previously (Carlson & Breslow, 1981a) although the difference in ΔH between the two peptides is slightly less than previously observed. Comparative $\Delta G°$ values for Phe-Phe-NH_2 and Phe-PheH are the same as those for Phe-Tyr-NH_2 and Phe-TyrH but with some indication of slightly different enthalpy–entropy relationships (see Discussion).

Comparison of the data in Tables I and II indicates reasonable agreement between van't Hoff and calorimetric values, with the exception of Phe-Tyr-NH_2. Thus, calorimetric and van't Hoff values for Phe-Phe-NH_2 and Ala-Tyr-NH_2 show a maximum deviation of 1.3 kcal/mol from each other, while values from the two methods for Phe-Tyr-NH_2 differ by 4.4 kcal/mol. The source of the discrepancy for Phe-Tyr-NH_2 is unclear, but, as discussed further below, similar comparative

[2] The pK_a of the nitrotyrosine of nitrated neurophysin II is 7.45 at 25 °C in the absence of peptide and 6.85 at complete saturation with peptide (Breslow & Weis, 1972). This in turn implies that binding of peptide to neurophysin in which the nitrotyrosine is ionized is 4 times stronger than to the protonated species. Thus, the observed binding constant at each temperature will contain a small contribution from binding to the ionized nitrotyrosine species which will vary with temperature according to the temperature dependence of nitrotyrosine ionization. Assuming the ΔH of nitrotyrosine ionization in neurophysin to be the same as that for phenol (6 kcal/mol), the nitrotyrosine pK_a effect can be shown to contribute less than 1 kcal/mol to the apparent van't Hoff ΔH of binding over the temperature range 10–40 °C and +20 cal/(deg·mol) to ΔC_p over the same temperature interval.

Table III: Temperature Dependence of $\Delta H_{binding}$ of Phe-Phe-NH_2 at pH 5.6: Batch Calorimetry Studies

protein[a]	buffer[a]	$\Delta H_{binding}$ (kcal/mol) at 15 °C	25 °C	35 °C	ΔC_p[b] (cal K^{-1} mol^{-1})
NP I	0.1 M KOAc	−5.4 ± 0.3	−7.2 ± 0.4	−8.5 ± 0.6	−163 ± 33[b]
NP II	0.1 M KOAc	−5.4 ± 0.1	−7.5 ± 0.7	−9.5 ± 0.7	−215 ± 34
NP I	0.1 M potassium phosphate	−5.4 ± 0.1	−7.5 ± 0.4	−8.9 ± 1.3	−178 ± 36
NP II	0.1 M potassium phosphate	−6.0 ± 0.2	−9.9 ± 0.4	−12.8 ± 1.4	−351 ± 44

[a] Final protein concentrations were 2 mg/mL throughout. Final peptide concentrations were 1.2 mM. All buffers contained 0.16 M KCl. [b] Errors calculated from errors in individual points according to Bevington (1969).

enthalpy trends can be discerned regardless of the experimental method.

Heat Capacities of Peptide Binding. The data in Figure 2 indicate that, when sufficient temperatures are examined, van't Hoff plots are nonlinear, with the change in slope indicating a negative heat capacity change associated with binding. Calculated values of ΔC_p from the van't Hoff data are listed in Table II. With the assumption that ΔC_p was independent of temperature, values of approximately −400 cal/mol were calculated for most peptides. The summed contribution of temperature effects on α-amino and nitrotyrosine pK_a values to the calculated ΔC_p is approximately 10%.[2,3]

A more direct and accurate calculation of ΔC_p for Phe-Phe-NH_2 was obtained by studying the temperature dependence of ΔH by batch calorimetry. Results from these studies using two solvents (0.1 M potassium phosphate and 0.1 M KOAc, both in 0.16 M KCl) and comparing the binding to both bovine neurophysin I and neurophysin II are shown in Table III. Comparison of the two buffer systems was of interest since phosphate has been shown to promote dimerization of neurophysin II (Tellam & Winzor, 1980). In both buffer systems, both ΔH and ΔC_p are slightly more negative for neurophysin II than for neurophysin I. Furthermore, phosphate appears to have a selective effect on neurophysin II; i.e., while the results for neurophysin I in the two buffers are essentially identical, substitution of phosphate for acetate produces a significant increase in the magnitude of the value of ΔC_p for neurophysin II. In view of the effects of buffer on neurophysin II, it is difficult to meaningfully compare the calorimetric and van't Hoff ΔC_p values. However, the van't Hoff ΔC_p for Phe-Phe-NH_2 (obtained in KCl–citrate) is essentially identical with the calorimetric ΔC_p obtained in KCl–phosphate but significantly larger than the calorimetric ΔC_p obtained in KCl–acetate.

Linkage between Peptide and Proton Binding. In another series of experiments, the enthalpy of binding Met-Tyr-Phe-NH_2 and in some cases Phe-Phe-NH_2 to neurophysins I and II was compared at pH 2.5, 6.2, and 8.1. The dependence of the apparent enthalpy of binding on the enthalpy of ionization of the buffer was also examined at pH 6.2 and 8.1. The pH 6.2 and 8.1 studies made use of the relationship $\Delta H_{app} = \Delta H_{binding} + \Delta\nu_H{}^+\Delta H_{ion}$ where $\Delta\nu_H{}^+$ corresponds to the moles of H^+ bound per mole of ligand, ΔH_{ion} is the enthalpy of ionization of the buffer, and $\Delta H_{binding}$ is the enthalpy of peptide binding corrected for the enthalpy of buffer ionization. These

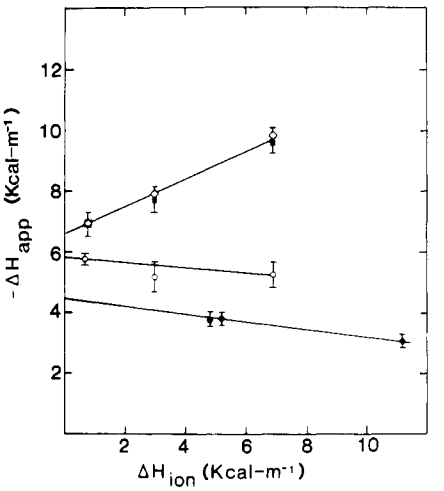

FIGURE 3: Dependence of ΔH_{app} for binding Met-Tyr-Phe-NH_2 and Phe-Phe-NH_2 to neurophysins I and II on pH and ΔH_{ion} of buffer. Since $\Delta H_{app} = \Delta H_{binding} + \Delta\nu_H{}^+\Delta H_{ion}$, the number of protons, $\Delta\nu_H{}^+$, whose binding is linked to binding of the ligand is given by the slope. $\Delta H_{binding}$, the enthalpy of binding corrected for buffer effects, is given by the y intercept. Ligand and protein concentrations were 1.2 mM and 1 mg/mL, respectively, in all experiments. Note that $\Delta H_{binding}$ is not corrected for incomplete peptide binding in this figure; corrections to the value at saturation are given in the text. Buffers were 20 mM and contained 160 mM KCl: (●) pH 8.1, Met-Tyr-Phe-NH_2 and neurophysin II in Tris, Hepes, and Epps [*N*-(2-hydroxyethyl)-piperazine-*N*′-3-propanesulfonic acid]; (○) pH 6.2, Met-Tyr-Phe-NH_2 and neurophysin I in citrate, Ada [*N*-(2-acetamido)-2-iminodiacetic acid], and Aces [*N*-(2-acetamido)-2-aminoethanesulfonic acid]; (■) pH 6.2, Met-Tyr-Phe-NH_2 and neurophysin II in citrate, Ada, and Aces; (◇) pH 6.2, Phe-Phe-NH_2 and neurophysin II in citrate, Ada, and Aces. Values of ΔH_{ion} (in kcal/mol) from Allewell et al. (1979) are as follows: Aces, −7.71; acetate, −0.09; Ada, −3.36; citrate, −0.803; Epps, −5.46; Hepes, −5.28; phosphate, −0.83; Tris, −11.27.

data allow the linkage between proton and ligand binding to be characterized [e.g., see Allewell et al. (1979)]. This information is of interest because of evidence indicating that electrostatic interactions are involved in ligand binding [e.g., see Breslow et al. (1971) and Breslow & Gargiulo (1977)].

With respect to the pH dependence of the enthalpy of binding, values of ΔH_{app} for both Met-Tyr-Phe-NH_2 and Phe-Phe-NH_2 in citrate and phosphate at pH 2.5 and a ligand concentration of 1.2 mM were in the range of −1.8 ± 0.7 kcal/mol of protein for neurophysin I and −3.2 ± 1.0 kcal/mol of protein for neurophysin II. However, these ligand concentrations are not sufficient to saturate the protein since the binding constant for peptide decreases markedly as the pH decreases. We can calculate the degree of protein saturation with peptide under these conditions using the pH dependence of the peptide binding constant as determined for Phe-Tyr-NH_2 (Breslow & Gargiulo, 1977); the pH dependence below pH 6 is determined by a protein carboxyl and therefore should be the same for all these peptides. Such calculations indicate that the concentrations of Phe-Phe-NH_2 and Met-Tyr-Phe-NH_2 used at pH 2.5 lead to 35% and 20% saturation, respectively, of the peptide binding site. Hence, the true values

[3] The contribution of α-amino protonation to the van't Hoff data is different from its contribution to the calorimetric data because the pK_a of the α-NH_3^+ is temperature dependent. The magnitude of the contribution is calculated by correcting the observed binding constant at each temperature to the binding constant for the completely protonated peptide, using a value of 10 kcal/mol for the ΔH of α-NH_3^+ ionization. The corrected binding constants are used to calculate the corrected ΔH of binding. The difference between corrected and uncorrected ΔH values represents the contribution of α-NH_2 protonation to the van't Hoff ΔH. The contribution of temperature effects on α-NH_3^+ ionization to the ΔC_p of peptide binding is −60 cal/(deg·mol).

Table IV: Thermodynamic Parameters at 25 °C for Bonding of Individual Peptide Segments to Neurophysin II[a]

segment	$\Delta G°$ (kcal/mol)	ΔH (kcal/mol) calorimetry	ΔH (kcal/mol) van't Hoff	ΔS (cal K^{-1} mol^{-1})	ref peptides
side chain β-CH_3, residue 1	−1.2	+2.1		+11.1	Ala-Tyr-NH_2, Gly-Tyr-NH_2
side chain $-C_\gamma H(CH_3)_2$, residue 1	−0.7	+1.3		+6.7	Leu-Tyr-NH_2, Ala-Tyr-NH_2
side chain $-CH_2SCH_3$, residue 1	−1.0	−2.4 (−1.4)[b]		−4.7 (−1.3)[b]	Met-Tyr-NH_2, Ala-Tyr-NH_2
side chain $-CH_2SCH_3$, residue 1	−1.1	−2.6 (−1.6)[b]		−5.0 (−1.7)[b]	Met-Tyr-Phe-NH_2, Ala-Tyr-Phe-NH_2
side chain −SS, residues 1, 6	−1.8	−7.6 (−2.6)[b]		−19.5 (−2.7)[b]	(Cys-Tyr-NH_2)$_2$, Ala-Tyr-NH_2
side chain phenyl ring, residue 1	−2.15 ± 0.15	−7.0 (−6.0)[b]	−3.9 (−2.6)[b]	−11.2 ± 5[c] (−7.2)[b,c]	Phe-Tyr-NH_2, Ala-Tyr-NH_2
side chain phenolic OH, residue 2	0	−6.3	−2.3	−14.4 ± 7[c]	Phe-Tyr-NH_2, Phe-Phe-NH_2
side chain phenolic OH, residue 2	+0.1		−0.2	−1	Phe-TyrH, Phe-PheH
carboxamide, residue 2	−2.0		−3.4	−4.7	Phe-Tyr-NH_2, Phe-TyrH
carboxamide, residue 2	−1.9		−1.3	+2.0	Phe-Phe-NH_2, Phe-PheH
$-CH(CH_2C_6H_5)CONH_2$, residue 3	−0.8	+1.3		+7.0	Met-Tyr-Phe-NH_2, Met-Tyr-NH_2
$-CH(CH_2C_6H_5)CONH_2$, residue 3	−0.7	+1.5		+7.4	Ala-Tyr-Phe-NH_2, Ala-Tyr-NH_2

[a] For each segment in column 1, the thermodynamic parameters were calculated as the difference in these parameters between the reference peptides in the last column. The data used for calculations were from Table I (calorimetric values) and Table II (van't Hoff values). [b] Values in parentheses are corrected for the contribution of α-NH_2 protonation as described in the text. [c] Represents ΔS calculated from the average of calorimetric and van't Hoff values of ΔH with the deviation representing the spread in ΔS values obtained if either ΔH value was used alone.

of ΔH_{app} are in the range of −7 ± 2 kcal/mol for neurophysin I and −12.5 ± 3.5 kcal/mol for neurophysin II. Because small errors in assumed binding constants at pH 2.5 will have a large impact on these calculations, these results must be considered tentative. Nonetheless, when compared with the enthalpies at pH 6.2 for binding Phe-Phe-NH_2 and Met-Tyr-Phe-NH_2 to neurophysins I and II in the same buffers (e.g., Table III and Figure 3), they are consistent with earlier results (Breslow & Gargiulo, 1977) indicating that binding enthalpies remain the same or become slightly more negative as the pH is lowered from 6.2 to 2.

At pH 8.1 (Figure 3), results extrapolated to a zero value for the heat of buffer ionization indicate a value of $\Delta H_{binding}$ of −4.5 kcal/mol for the binding of Met-Tyr-Phe-NH_2 to neurophysin II at a ligand concentration of 1.2 mM. When the additional necessary correction is applied for the decreased binding affinity at pH 8.1 [expected to be one-ninth that at pH 6.2 because of ionization of the peptide α-NH_3^+ with a pK_a of 7.15 (Breslow et al., 1971, 1973)], this value of $-\Delta H$ is increased to approximately 11 kcal/mol. The more negative value at pH 8.1 relative to the similarly extrapolated value at pH 6.2 (−5.8 kcal/mol in Figure 3 corrected to −6.5 kcal/mol at complete binding) is generally compatible with the expected release of heat associated with the necessary protonation of the peptide on binding to neurophysin at pH 8.1 [e.g., see Breslow et al. (1971)].

The relationship between the corrected extrapolated binding enthalpies at pH 2.5, 6.2, and 8.1 is reasonable with respect to the proposed mechanism of the binding interaction, which includes formation of a salt bridge between the protonated peptide α-amino and an unprotonated neurophysin carboxyl (see Discussion), but this is not true of some of the other data in Figure 3. Consideration of the salt bridge as the principal factor determining changes in proton equilibria associated with the peptide–protein interaction (for which there is considerable evidence) leads to the conclusion that only trivial changes in proton equilibria (≤0.1 mol of H^+/mol) should accompany binding of Met-Tyr-Phe-NH_2 and Phe-Phe-NH_2 at pH 6.2. However, while the flat slope of the pH 6.2 line for neurophysin I in Figure 3 is in agreement with this conclusion, the pH 6.2 data for neurophysin II in Figure 3 suggest the release of approximately 0.4 mol of H^+ per mole of bound peptide. The neurophysin II data are particularly surprising since pH titration of Met-Tyr-Phe-NH_2 addition at pH 6.2 indicates no proton release (E. Breslow, unpublished results). Moreover, differences between neurophysins I and II at pH 6.2 cannot be explained by the presence of a histidine in neurophysin I since the pK_a of this histidine is known to be slightly lowered by peptide binding (Cohen et al., 1972), a fact which should lead to the release of 0.05–0.1 more protons by neurophysin I than by neurophysin II at pH 6.2. Finally, binding of Met-Tyr-Phe-NH_2 at pH 8.1 should be accompanied by the binding of approximately 0.9 mol of H^+ per mole (see above), and this value has also been confirmed experimentally (Breslow et al., 1971). However, the slope of the pH 8.1 line in Figure 3, corrected to complete binding, suggests a maximal proton uptake by neurophysin II of 0.3 mol of H^+ per mole. Possible sources of these discrepancies are discussed below.

Discussion

Study of the binding to neurophysin of systematically related peptides has provided a picture of a number of bonding interactions between peptides and protein at the strong hormone binding site [for the most recent review, see Breslow (1984)]. As shown in Figure 1, these are the following: (1) a strong salt bridge between the protonated peptide α-amino and a neurophysin carboxyl; (2) a strong and highly specific apolar interaction involving the aromatic ring in position 2 of the peptide; (3) participation of the side chains of both residues 1 and 3 in apolar interactions with the protein; (4) probable hydrogen bonding to the protein of the peptide backbone between residues 2 and 3 (i. e., the carboxamide terminus of bound dipeptides). Because all peptides appear so far to produce the same structural changes in the protein on binding [e.g., see Breslow et al. (1973)], these studies have also allowed the free energy contributions of bonding several individual segments of the peptide to be evaluated (as a first approximation) by calculation of binding affinity differences between systematically related peptides (Breslow, 1984). For example, the binding free energy difference between Gly-Tyr-NH_2 and Ala-Tyr-NH_2 is taken to represent the free energy of formation of the bond between the alanine β-CH_3 and protein. This bonding free energy is analogous to the "intrinsic" binding energy of Jencks (1981) and is independent of mole fraction.

The calorimetric and van't Hoff data obtained here can be analyzed as above to obtain the enthalpic contribution of individual bonding interactions and, using free energy data, the entropic contribution as well. The data are shown in Table IV and show the following characteristics. For the side chain of residue 1, segments containing only methyl or methylene carbon atoms make a positive contribution to the enthalpy and entropy of binding. This is the "expected" contribution for apolar interactions [e.g., see Ross & Subramanian (1981)]. Alternatively, aromatic and sulfur-containing side chains in

position 1 appear to make a negative contribution to these parameters. These apparent (uncorrected) negative ΔH contributions range from −2.4 to −7.6 kcal depending on the particular side chain considered. However, these values must be corrected for the enthalpic contribution of protonation of the peptide α-NH_2 since only the protonated form of the peptide binds and not all the peptides used for comparison here are completely protonated at pH 6.2. For example, the α-NH_3^+ pK_a of peptides with glycine or aliphatic side chains at residue 1 is approximately 7.9 at 25 °C (Breslow et al., 1971), and their state of protonation is therefore essentially unaffected by binding at pH 6.2. However, for peptides with a methionine or phenylalanine in position 1, the α-NH_3^+ pK_a is 7.15–7.2 at 25 °C (Breslow et al., 1971, and unpublished results). These peptides therefore bind 0.1 H^+ per mole when complexing with neurophysin. Since $\Delta H_{ionization}$ at 25 °C is expected to be +10 kcal/mol (Steinhardt & Beychok, 1964), −1 kcal/mol of the apparent enthalpy of a phenyl ring or $-SCH_3$ in position 1 may be attributed to α-NH_2 protonation. (For the van't Hoff data, the correction can be shown to be 1.3 kcal/mol because of the different temperatures at which the data are obtained.)[3] For the disulfide of (Cys-Tyr-NH_2)$_2$, the enthalpic contribution of α-NH_2 protonation is much greater than 1 kcal/mol because this peptide is only half-protonated at pH 6.2 and the apparent, –SS– contribution of −7.6 kcal should therefore probably be reduced to approximately −2.6 kcal. The corrected ΔH and ΔS values for residue ΔH values that required this correction are given in parentheses in Table IV adjacent to the uncorrected values. Given the above corrections, the enthalpic contributions of the CH_2SCH_3, –SS–, and phenyl groups at position 1 range from −1.5 to −6 kcal/mol, the contribution of the phenyl group, as measured calorimetrically, being particularly significant. These results support the conclusions of Ross & Subramanian (1981) from model systems that van der Waals interactions associated with the apolar binding of polarizable atoms or residues can lead to negative enthalpy and entropy changes.[4]

In position 2 of peptide ligands, the data indicate the carboxamide terminus, corresponding to the backbone region between residues 2 and 3 of the hormones, also contributes negative enthalpies of between 1.4 and 3.5 kcal/mol, the apparent contribution depending somewhat on the peptides compared as discussed further below. (Note that no correction for α-NH_2 protonation need be applied here because all the peptides used for thermodynamic comparison have the same pK_a.) This is the expected contribution of hydrogen bonds in a nonpolar environment (Ross & Subramanian, 1981) and is in accord with earlier conclusions (Carlson & Breslow, 1981a) on hydrogen bonding of this region of the peptide. Another apparent contributor at position 2 to the negative enthalpy change on binding is the tyrosine –OH. Calorimetric comparisons of Phe-Tyr-NH_2 and Phe-Phe-NH_2 (Table IV) indicate a ΔH value of −6.3 kcal/mol for bonding of the tyrosine –OH, while van't Hoff data support a significantly smaller but still negative value of ΔH. The differences between the two sets of experiments again reflect the differences in the data for Phe-Tyr-NH_2. The apparent negative enthalpic contribution of this tyrosine –OH is compatible with fluorescence data indicating that Tyr-2 fluorescence is markedly quenched on binding (Sur et al., 1979) and suggesting therefore that the –OH might be hydrogen bonded to protein. This interpretation is supported by the fact that substitution of *p*-azidophenylalanine for phenylalanine in position 2 blocks binding to neurophysin (J. Carlson and E. Breslow, unpublished results), in turn suggesting that the para position is not exposed to solvent. The markedly slower ring rotation of Tyr relative to Phe in position 2 of bound peptides (Blumenstein et al., 1982) is also compatible with hydrogen bonding of the Tyr OH. Nonetheless, the small negative van't Hoff ΔH calculated from a comparison of Phe-Tyr-NH_2 and Phe-Phe-NH_2 becomes even smaller when Phe-TyrH and Phe-PheH are compared (Table IV). While we are reluctant to overinterpret this trend in view of the small differences involved and the lack of corroborating calorimetric data, the results tentatively suggest that the enthalpic contribution of the tyrosine –OH may be dependent on interactions of the terminal carboxamide. A mutual thermodynamic interaction between the terminal carboxamide and the phenolic –OH in position 2 is also suggested by the smaller apparent enthalpic contribution of the carboxamide in Phe-Phe-NH_2 relative to Phe-Tyr-NH_2 (see Table II).

In contrast to the negative bonding enthalpies of the phenyl group in position 1 and the carboxamide in position 2, the net enthalpic contribution of a phenylalanylamide residue in position 3 is weakly positive. This suggests that the carboxamide in position 3 is not hydrogen bonded and that the phenyl group in position 3 interacts differently with the protein than that in position 1. In fact, NMR studies of the binding of tripeptides to neurophysin (Balaram et al., 1973) suggest that the phenyl ring in the side chain of position 3 is not markedly affected by binding and that the principal segment of the position 3 side chain that binds is the β-CH_2. This is in contrast to the phenyl side chain in position 1, which is suggested by preliminary NMR studies (V. Sardana and E. Breslow, unpublished results) to be markedly restricted by binding. The observed positive values of ΔH and ΔS for position 3, which are similar to those noted above for binding of other aliphatic groups, are therefore in accord with the NMR observations.

The above results therefore point to two sources which contribute to the negative enthalpy change associated with the neurophysin–peptide interaction: tight hydrophobic bonding involving polarizable atoms, the negative enthalpy here probably arising from van der Waals interactions (Ross & Subramanian, 1981),[4] and hydrogen bonding. Binding-induced conformational changes within the protein dimer may also be associated with a negative ΔH. However, changes in the extent of protein dimerization per se appear not to contribute significantly to the observed negative values of ΔH. Nicolas et al. (1980) have reported the ΔH of neurophysin dimerization as zero, although our own analysis of their published results leads to a value of −3 kcal/mol. With the exception of neurophysin II in phosphate buffer (vide infra), the weight fraction of neurophysin dimer can be shown to change from approximately 50% in the absence of peptide to 100% dimer in the presence of peptide under our conditions [e.g., see Cohen et al. (1979)]. Therefore, by these calculations, the maximum negative enthalpic contribution of dimerization to our data is 1.5 kcal/mol. The data in Table III also support a negligible contribution of increased dimerization to the negative enthalpy of peptide binding. The dimerization constant of unliganded neurophysin II in phosphate is higher than in acetate (Tellam & Winzor, 1980); Whittaker & Allewell, 1985). Under the conditions in Table III, unliganded neurophysin II in phosphate at 25 °C is 85% dimer by weight; therefore, peptide-induced

[4] Another view of the negative enthalpy change associated with binding the more polarizable side chains is that "stiffening" of the vibrational modes in the complex (relative to those of the unliganded molecules) is increased with the more polarizable side chains (Sturtevant, 1977). These two explanations are not necessarily different, and our data do not permit a distinction between the two.

changes in dimerization are small relative to those in acetate. Nonetheless, the enthalpy of peptide binding in phosphate is more negative than in acetate. By these same arguments, the data in Table III argue that the negative heat capacity change associated with peptide binding does not reflect the increased dimerization; i.e., the heat capacity change for neurophysin II is more negative in phosphate than in acetate.

The observed overall thermodynamic values for peptide–protein ligation represent the sum of multiple, often thermodynamically opposing, phenomena associated with the reaction [e.g., see Breslow (1984)]. In this context, bonding of the side chain in position 1 and hydrogen bonding of the phenolic hydroxyl in position 2 and of the backbone between residues 2 and 3 are unlikely to represent the sole exothermic bonding interactions between neurophysin and peptide. For example, the phenyl ring at peptide position 2 contributes a *minimum* of −4.4 kcal in free energy to binding (Breslow, 1984); by analogy with the phenyl ring at position 1, this bonding may also occur with a significant release of heat. Interactions of the peptide α-NH_3^+ group with neurophysin are thermodynamically complex, but the purely electrostatic component of salt-bridge formation can be calculated from existing data (Breslow & Gargiulo, 1977) not to involve the release of heat. However, there is probably an additional hydrogen bonding component to interaction of the α-NH_3^+ [e.g., see Breslow (1984)] and this also has the potential for contributing to the negative enthalpy change.

Of the data obtained here, the most difficult to reconcile with earlier work are calorimetric ΔH values for peptide binding to neurophysin II as a function of pH and buffer. In particular, enthalpy changes associated with buffer changes at constant pH lead to values for proton uptake or release by the peptide neurophysin II system that differ both from expected values and from values obtained by direct titration. By contrast, neurophysin I data, although available only at one pH, were in accord with predicted values. A potential explanation of these results is that they reflect interactions between neurophysin II and buffer that are different in the free and bound states, that are not available to neurophysin I and that are dependent on the nature of the buffer. Direct evidence for this, under limited conditions, is found in the different effects of phosphate and acetate on neurophysin II and neurophysin I in Table III. The results underscore the fact that neurophysins I and II may exhibit significant behavioral differences despite their high degree of homology. A difference between these neurophysins in affinity for bromophenol has also been reported recently (Carlson & Breslow, 1981b). The structural basis and functional consequences of these differences await investigation.

ACKNOWLEDGMENTS

We thank Dr. Irwin Chaiken for the affinity chromatography experiments and Vicki Edge for purifying some of the protein.

Registry No. Phe-Tyr-NH_2, 38678-74-9; Met-Tyr-NH_2, 38102-15-7; Met-Tyr-Phe-NH_2, 37637-13-1; Phe-Phe-NH_2, 15893-46-6; Ala-Tyr-NH_2, 38678-78-3; (Cys-Tyr-NH_2)$_2$, 52329-45-0; Gly-Tyr-NH_2, 3715-41-1; Leu-Tyr-NH_2, 17263-43-3; Ala-Tyr-Phe-NH_2, 37637-11-9; Phe-TyrH, 78123-48-5; Phe-PheH, 35402-94-9.

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Formation of Microtubules at Low Temperature by Tubulin from Antarctic Fish†

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Received September 5, 1984; Revised Manuscript Received December 10, 1984

ABSTRACT: Tubulin was isolated from two species of antarctic fish, *Pagothenia borchgrevinki* and *Dissostichus mawsoni*, by cycles of temperature-dependent assembly, centrifugation, disassembly, and centrifugation. The preparations were found to consist almost entirely of tubulin and to contain negligibly small amounts of microtubule-associated proteins. This tubulin polymerized to make microtubules of ordinary dimensions. The formed microtubules appear to be in labile equilibrium with free tubulin dimer at all temperatures observed. In a buffer consisting of 0.1 M 1,4-piperazinediethanesulfonic acid, 2 mM dithioerythritol, 1 mM $MgSO_4$, 2 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid, and 1 mM guanosine 5′-triphosphate, pH 6.9, the tubulin of *P. borchgrevinki* has a critical concentration for assembly of 0.046 (±0.008) mg/mL at 35 °C and 0.74 (±0.15) mg/mL at the habitat temperature of the fish, −1.8 °C. The critical concentration measured at the lower temperature is quite small relative to the critical concentration for formation of mammalian microtubules from pure tubulin at the same temperature, which must be at least 2 orders of magnitude larger. The antarctic fish microtubules may thus be called "cold stable" by comparison with mammalian microtubules. They do not fully dissociate at temperatures near 0 °C because they are composed of tubulin that assembles more readily at these temperatures than does mammalian tubulin. There is no evidence for the presence of a cold-stabilizing factor in association with the tubulin. These findings suggest that alteration of tubulin may be a means by which some poikilotherms can adapt to a cold environment. A van't Hoff plot of the apparent association constants measured between −1.2 and 35 °C was linear and yielded an apparent $\Delta H°$ of +13.7 kcal/mol and an apparent $\Delta S°$ of +74 eu for the addition of a fish tubulin subunit to the end of a growing microtubule.

Microtubules formed in vitro from the tubulin of mammals and birds are observed to disassemble to yield free tubulin dimers at temperatures near 0 °C. The free dimers can reassemble spontaneously to form microtubules at temperatures near 37 °C. [For reviews, see Timasheff & Grisham (1980) and Correia & Williams (1983).] In contrast, the microtubules present in the tissues of poikilotherms that live in cold habitats must be assembled from tubulin dimers at low temperatures, and they must remain assembled under those conditions. The fishes of the polar seas present an extreme example of such organisms: the two species dealt with in this paper, *Pagothenia borchgrevinki* and *Dissostichus mawsoni*, live perennially at temperatures near −1.8 °C. This paper describes the temperature dependence of assembly of tubulin from these fishes.

Microtubules that are "cold stable" have been described both in mammals and in cold-living poikilotherms. In mammalian cells, there appear to be different populations of cytoplasmic microtubules that can be differentiated on the basis of their resistance to depolymerization at low temperatures. Brinkley & Cartwright (1975), working with PtK_2 cells, have shown that at metaphase the kinetochore to pole microtubules do not depolymerize at temperatures near 0 °C although the adjacent interpolar microtubules do. Jones et al. (1980), in an electron microscopic study of neurons of rat brain, found that numerous intact microtubules were present after incubation of tissue slices for 1 h at 0 °C. A series of experiments with isolated microtubles led them to hypothesize that resistance to disassembly at low temperatures is conferred on tubulin by the presence of a non-tubulin factor of unknown identity. When microtubules were isolated from mouse brain by a cycle of

† This work was supported by Research Grant GM 25638 from the National Institutes of Health (to R.C.W.), by grants from the Vanderbilt University Research Council and the Vanderbilt University Natural Sciences Committee (to R.C.W.), and by Grant DPP81-16917 of the National Science Foundation (to A.L.D.).

0006-2960/85/0424-2790$01.50/0