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Optical Activity of Bovine Neurophysins and Their Peptide Complexes in the Near Ultraviolet*

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Abstract. Circular dichroism studies of the bovine neurophysins in the near ultraviolet show a strong negative band at 280 nm and a strong positive band at 248 nm, both of which are attributable almost exclusively to disulfide transitions. The ellipticities per disulfide bond of the unresolved bands in neurophysin-II are -2900 deg cm^2/decimole and $+2300$ deg cm^2/decimole at 280 nm and 248 nm, respectively. Binding of oxytocin, vasopressin, or the peptide *S*-methyl-L-cysteinyl-L-tyrosyl-L-phenylalanine amide lead to large changes in optical activity in the near and far ultraviolet. Of these, circular dichroism changes above 290 nm are attributed to changes in the optical activity of neurophysin disulfides, while changes elsewhere are more generally ascribed to changes in either disulfide, tyrosine, or peptide bond transitions. Optical rotatory dispersion studies show that calcium ion, at concentrations of 0.01 M, has only trivial effects on the affinity of bovine neurophysins for oxytocin.

The neurophysins are a group of closely related proteins found in the hypothalamus and posterior pituitary glands of mammals (and some lower organisms) in noncovalent combination with the hormones oxytocin and vasopressin. Amino acid analyses[1] and binding studies[2] indicate that, to the nearest integer, each of the major bovine neurophysins binds one mole of either oxytocin or vasopressin per 10,000 g. Molecular weight studies in this laboratory[3] and elsewhere[1,4] indicate that the bovine neurophysins have a monomeric molecular weight of approximately 10,000 and reversibly aggregate to higher species.

The optical activity of the neurophysins is of interest on several counts. With respect to amino acid composition, the neurophysins are unusual in that they are characterized by a very high disulfide content—6 to 7 disulfide bonds per mole (10,000 g)[1] and a low content of most aromatic amino acids—no tryptophan, 1 tyrosine, and 3 phenylalanines per mole.[1,5] The high disulfide composition is reflected in the absorption spectra of the neurophysins, whose $A_{260}/_{280}$ ratios are greater than unity,[6] and suggests that the neurophysins are unique systems for the observation of disulfide optical activity. In addition, marked solubility changes in neurophysin upon combination with oxytocin and vasopressin[7] suggest that conformational changes in the protein may result from the protein–hormone interaction; studies of the relative optical activity of neurophysin and its complexes are appropriate to probe the possible existence of such a change. Finally, calcium ion has been reported to block the protein–hormone interaction.[8,9] A

subsidiary purpose of this study is to investigate any effects of calcium ion on the optical activity of either neurophysin or its hormone–complexes.

Materials and Methods. The crude hormone–neurophysin complexes were prepared from acetone-dried bovine posterior-pituitary powder (a generous gift from Parke Davis and Co.) according to the procedure of Hope.[10] Subsequent separation of the hormones from the neurophysins, and resolution of the individual neurophysins, were performed as previously described.[2] The isolated fractions were lyophilized twice to remove pyridine–acetate buffer used in the purification, or lyophilized once and then deionized on a Dintzis column.[11] Purified neurophysins-I and -II were homogeneous on disc electrophoresis at pH 9.5[2] and were essentially identical in amino acid composition to values reported by Rauch *et al.*[1] The exceptions are that we find 14 (not 12) half-cystines, 7 (not 6) arginines, and 7 (not 8) NH_3 in neurophysin-II, and 14 (not 12) half-cystines and 5 (not 6) NH_3 in neurophysin-I.

Oxytocin was a gift from Prof. V. du Vigneaud and Dr. D. Yamashiro and lysine vasopressin was a gift from Prof. R. Walter. *S*-methyl-L-cysteinyl-L-tyrosyl-L-phenylalanine amide was purchased from Cyclo Chemical Corp. and was titrimetrically and spectrophotometrically pure after removal of a water-insoluble contaminant. Dithiothreitol was from Calbiochem. Poly-α-L-glutamic acid (mol wt = 175,000) was from Mann Research Lab. and was donated by Dr. M. Sonenberg. All other reagents were Reagent Grade, and only deionized water was used.

Optical rotatory dispersion (ORD) and circular dichroism studies were performed at room temperature on a Cary Model 60 spectropolarimeter equipped with a model 6001 circular dichroism (CD) accessory. The CD attachment was calibrated with *d*-10-camphor sulfonic acid as specified by Cary Instruments. Helical poly-α-L-glutamic acid gave a residue ellipticity (uncorrected for refractive index) of 42.5×10^3 deg cm^2/decimole at pH 4.2, in good agreement with the literature.[12,13] Studies of neurophysin and its complexes were performed at pH values no lower than 6.2 to circumvent the large decrease in solubility occurring at lower pH values upon complex formation. Protein concentrations were 2 mg/ml in 0.16 M KCl; optical path lengths were 1 cm for near UV studies and 1 mm in the far UV. Unless otherwise specified, the results of circular dichroism studies are reported as the ellipticity $[\theta]$ per mole of protein and are uncorrected for refractive index.

pH was determined with a Radiometer model 4 pH meter. Spectrophotometric studies were performed using a Beckman model DU spectrophotometer.

Results. Circular dichroism of neurophysin: In Fig. 1, the near ultraviolet CD spectra of neurophysin-II under a variety of conditions are shown. At neutral pH (Fig. 1*A*) two principal bands are seen—a negative band centered at 280 nm and a positive band at 248 nm. Between 240 and 200 nm (not shown) is a compound-negative band with a shoulder near 225 nm and a maximum intensity at 205 nm. The ellipticities *per peptide bond*, uncorrected for refractive index, are -2400 deg cm^2/decimole at 223 nm and -5200 deg cm^2/decimole at 205 nm and indicate a low apparent α-helix content. Although the far UV data are clearly of interest, the two near UV peaks will be emphasized here. They are also seen in neurophysin-I, although the maximum ellipticity of the 248 nm peak in NP-I is 30% greater than in NP-II, and they appear to contain little or no contribution from tyrosine. Thus, increasing the pH from 6 to 11.3 (Fig. 1*A*) is without significant effect on either the 248 nm or 280 nm band of the neurophysins although the lone tyrosine of neurophysin-II is more than $^2/_3$ ionized at pH 11.3 under the conditions of the experiment. The extent of tyrosine ionization at pH 11.3 can be gauged from the absorption difference spectrum between the samples at pH 11.3 and 6.2 shown in the inset in Fig. 1*A*. The spectrum

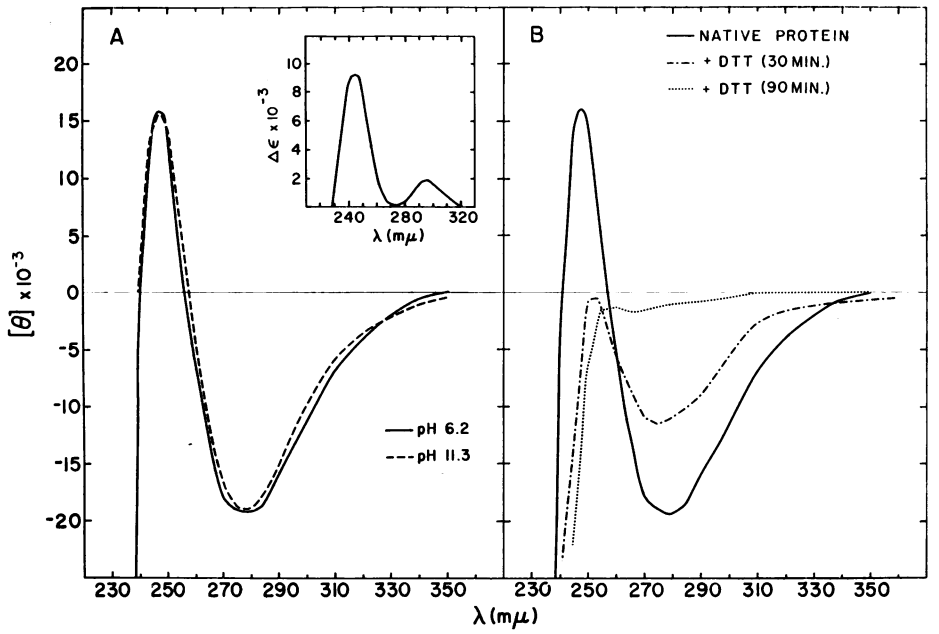

FIG. 1. *A*. CD spectra of neurophysin-II at pH 6.2 and 11.3. The inset shows the difference in the absorption spectrum of NP-II between pH 11.3 and pH 6.2. *B*. CD spectrum of NP-II alone and in the presence of 0.02 M dithiothreitol, at pH 6.2, at the time intervals noted.

is a typical tyrosine-ionization difference spectrum and the magnitude of both the 245 and 295 nm peaks indicate that ionization is at least 70% complete.[14,15] Because there is no tryptophan in the neurophysins, and because phenylalanine transitions do not occur at 280 nm, and are weaker and characterized by a degree of fine structure[16] not seen here, the lack of effect of tyrosine ionization on these bands indicates that they almost exclusively represent disulfide transitions. This is supported by the effect of dithiothreitol (Fig. 1*B*) which abolishes both bands, although at unequal *apparent* rates. Identical results were obtained with mercaptoethanol (although reduction was slower than with dithiothreitol) and by prolonged exposure to pH 12, the higher pH favoring both -S-S- interchange and oxidation.

Optical rotatory dispersion of neurophysin and its complexes and the effect of calcium ion: Fig. 2 shows the near ultraviolet ORD spectrum of neurophysin-II alone and in the presence of one equivalent of oxytocin. At pH 6.2 marked differences prevail between the spectrum of NP-II in the presence of oxytocin and the theoretical additive sum of NP-II and oxytocin, indicating that complex formation perturbs one or more chromophores on neurophysin and/or oxytocin. Qualitatively similar effects are noted on binding of oxytocin to NP-I and on binding of lysine vasopressin to NP-II.[17] The sensitivity of the ORD spectrum to the degree of neurophysin–oxytocin interaction permits an estimate of the effect of calcium ion on this interaction. At pH 7.7, an increased levorotation of the 1:1 neurophysin–oxytocin mixture occurs (Fig. 2) and can

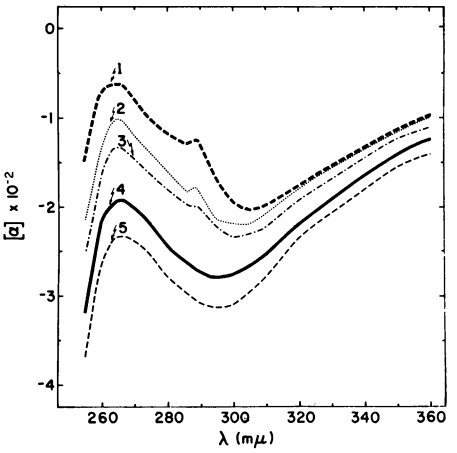

FIG. 2. ORD spectra of NP-II alone, and in the presence of 1 eq of oxytocin. (1) NP-II + oxytocin, pH 6.2; (2) NP-II + oxytocin, pH 7.7; (3) NP-II+ oxytocin + 0.01 M $CaCl_2$, pH 7.7; (4) NP-II alone, pH 6.2; (5) theoretical additive sum of NP-II + oxytocin, pH 6.2 (calculated from the individual spectra of the same protein and hormone solutions used to form the complex).

be shown to arise from an increased dissociation of the complex as the pH is raised.[18] Even under these conditions of partial dissociation (which will be sensitive to small changes in binding constant), 0.01 M Ca^{++} produces only a trivial increase in the degree of dissociation (Fig. 2). From the data, the change in binding constant can be estimated as less than twofold. It is significant also that 0.01 M Ca^{++} has no effect on the ORD of NP-II alone in this wavelength region, and that preincubation of NP-II with 0.01 M Ca^{++} does not affect the ORD changes associated with oxytocin binding.

Circular dichroism of neurophysin complexes: Individual transitions affected by neurophysin-hormone interaction can be located by CD studies. The CD spectrum of the complex differs from that of an unreacted mixture of neurophysin and oxytocin in three ways (Fig. 3*A*). First, an increased negative ellipticity is observed in the complex above 290 nm and is accompanied by the appearance of a small negative band at 291 nm. Secondly, there is a decrease in the magnitude of the negative band between 288 and 260 nm upon interaction. While this decrease may be solely the result of the increased positive ellipticity near 248 nm (see below), preliminary resolution of the individual CD bands indicates that the 248 nm band has little impact on ellipticities above 275 nm; this suggests real changes in transitions centered near or between 275 and 288 nm. Finally, there is an increased positive ellipticity near 248 nm which appears to be centered at somewhat shorter wavelengths. Thus, the baseline crossover wavelength near 240 nm is shifted approximately 5 nm to shorter wavelengths in the complex relative to the non-interacted system, and ellipticity differences between the complex and the non-interacted system increase from 12,000 deg cm^2/decimole at 245 nm to 18,000 deg cm^2/decimole at 240 nm. Although the exact center of this increased ellipticity is difficult to ascertain because of the steepness of the CD curves in this region, preliminary studies in the far UV suggest that it is centered at 237 nm. (In addition, far UV studies indicate a distinctly separate ellipticity difference of approximately 200,000 deg cm^2/decimole between the complex and the non-interacted system which appears to be centered at 205 nm.)

Qualitatively similar near UV changes to these shown in Fig. 3*A* occur upon interaction of oxytocin with NP-I and of lysine vasopressin with neurophysin-II[17]. Other peptides have also been shown to bind to neurophysin;

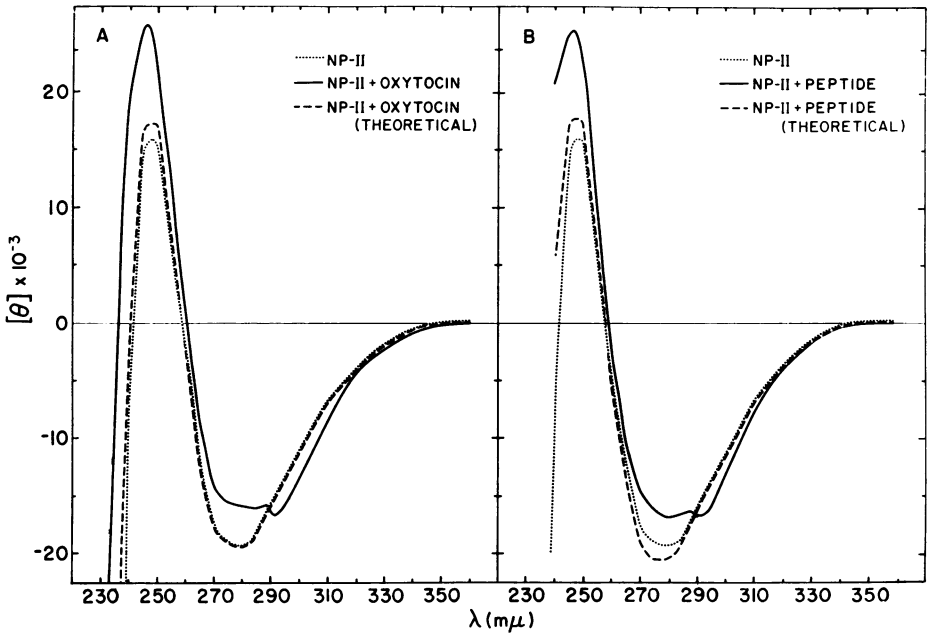

FIG. 3. *A*. CD spectra of NP-II in the presence of 1 eq of oxytocin at pH 6.2, compared to that of NP-II alone, and to the theoretical additive sum of NP-II and oxytocin. *B*. CD spectra of NP-II in the presence of 10^{-3}M *S*-methyl-Cys-Tyr-Phe-amide, pH 6.2. Also shown are NP-II alone and the theoretical additive sum of NP-II plus 10^{-3}M *S*-methyl-Cys-Tyr-Phe-amide.

these include *S*-methyl-Cys-Tyr-Phe-amide,[19] which binds with an affinity approximately 1/100 that of oxytocin. In Fig. 3*B* the CD spectrum of NP-II in the presence of *S*-methyl-Cys-Tyr-Phe-amide is shown. It is clear that binding of the peptide affects the circular dichroic spectra in the near UV in a qualitatively identical manner as does binding of oxytocin.

Discussion. Bond assignments in neurophysin: Identification of the positive dichroic band at 248 nm in neurophysin as a disulfide transition is fully compatible with known disulfide properties. Although this band overlaps with two neighboring transitions, preliminary curve resolution indicates that its true position is at 245 nm, a wavelength which at neutral pH is readily associated only with disulfide transitions.[20] A negative disulfide band is seen in L-cystine at 252 nm[20] and a positive disulfide band near 245 nm is observed in oxytocin and most of its analogs.[21,22]

Similarly, by the process of elimination, the negative band in neurophysin at 280 nm can be assigned only to a disulfide transition. (The only alternative not strictly precluded is the possibility of an as yet unidentified prosthetic group on neurophysin, which has an optically active transition at or near 280 nm. This appears unlikely because the molar extinction of the neurophysins at 280 nm is accounted for by their amino acid composition alone.[3]) Preliminary resolution of the spectra indicates that this band is little affected by overlap with the other bands, and is truly located at 277 nm, with a molar ellipticity of −20,000 deg

cm^2/decimole. Optically active disulfide transitions near 280 nm have been observed in oxytocin and its analogues,[21] and recent studies have suggested that almost half of the negative 280 nm dichroism band in RNase is due to optically active disulfide transitions.[23]

The most striking feature of the 280 nm band, however, is its intensity. The average negative ellipticity per disulfide bond (assuming 7 disulfides per mole) is almost 3000, which is greater by a factor of six than the intensity of the disulfide transition at this wavelength in oxytocin analogues which contain disulfides as the sole chromophore.[21] Thus, this band appears to represent the most intense protein disulfide transition, clearly identifiable as such, yet reported at this wavelength. However, the observed ellipticity per disulfide bond in neurophysin may not be unique. If, as suggested,[23] half of the 280 nm ellipticity in RNase is disulfide, then the ellipticity per disulfide bond in both neurophysin and RNase can be shown to quite similar.

The question arises as to whether the disulfide transitions at 248 nm and those at 280 nm represent transitions of the same or of different disulfide bonds. With respect to this, it is important to note that apparent differences in the rates of reduction of the 248 nm and 280 nm bands (Fig. 1) may be an artifact produced by the substantial overlap between the 248 nm band and both of its neighbors. Linderberg and Michl[24] have suggested that a single disulfide might show two near UV bands in the presence of asymmetric perturbations, or when the disulfide dihedral angle deviates from 90°.

Ellipticity changes accompanying complex formation: Changes in circular dichroism arising from neurophysin–hormone interaction have several possible explanations, some of which can be immediately precluded. First the increased negative ellipticity between 291 nm and 320 nm is most reasonably attributed to changes in disulfide transitions since neither phenylalanines[20] nor tyrosine model compounds[20,23] normally exhibit dichroism above 295 nm at neutral pH. That the disulfide(s) principally responsible for this increased negative ellipticity belongs to neurophysin and not to the hormones is clearly seen from the identical CD changes wrought by both oxytocin and *S*-methyl-L-cysteinyl-L tyrosyl-L-phenylalanine amide. In addition, the seemingly identical changes caused by both the peptide and by oxytocin suggest that there is no major contribution of the oxytocin disulfide to ellipticity changes elsewhere in the spectrum, either. This lack of a significant optical contribution from the oxytocin disulfide on binding stands in contrast to an apparent disulfide contribution to binding affinity.[19]

The decreased negative ellipticity between 275 and 288 nm that accompanies hormone–neurophysin interaction can potentially be attributed to either neurophysin disulfides or to altered contributions from the tyrosine of either the hormones or of neurophysin. Tyrosines are particularly probable contributors in that the tyrosine ring of the hormones participates in binding,[2] and difference spectra[4] suggest that binding perturbs a tyrosine on neurophysin. Ellipticity changes below 250 nm can, at this time, only be generally ascribed to either disulfides, tyrosines, or peptide bonds. (The magnitude of the changes makes phenylalanines improbable as significant contributors.) One potential source of

ellipticity changes both above and below 250 nm—the protonation of the α-NH_2 of the hormones upon binding[2]—can be precluded; large changes in ellipticity upon protonation of the hormone α-NH_2 ($pK_a = 6.3$)[25] do occur,[21] but the effects are negligible at 250 nm relative to those resulting from binding and can similarly be shown to be both negligible and in the wrong direction at 280 nm. The changes seen here throughout the spectrum must arise then either from direct perturbation of specific chromophores upon interaction, or from conformational changes that may be associated with binding.

Finally, a comment on the small effect of calcium ion is in order. Data presented here indicate that 10^{-2} M Ca^{++} maximally produces only a twofold change in the affinity of bovine NP-II for oxytocin. This lack of significant Ca^{++} effect has also been confirmed by potentiometric titration,[3] which indicates that 10^{-2} M Ca^{++} does not significantly bind to neurophysin nor affect changes in proton equilibria accompanying neurophysin-hormone interaction. These small or non-existent effects of Ca^{++} are thus in contrast with the report by Ginsburg and Ireland[18] that 10^{-6} M Ca^{++} completely blocks the interaction of porcine neurophysin with oxytocin, and shed serious doubt on the suggestion[18] that increases in Ca^{++} concentration *in vivo* play a direct role in the release of oxytocin by dissociating the hormone-neurophysin complex.

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Abbreviations: NP, neurophysin; *S*-methyl-Cys-Tyr-Phe-amide, *S*-methyl-L-cysteinyl-L-tyrosyl-L-phenylalanine amide; CD, circular dichroism; ORD, optical rotatory dispersion; DTT, dithiothreitol.

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