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Contribution of Tyrosine to Circular Dichroism Changes Accompanying Neurophysin–Hormone Interaction†

Esther Breslow* and Jane Weis

ABSTRACT: The contribution of changes in tyrosine ellipticity to circular dichroism changes accompanying neurophysin–hormone interaction was assessed by replacing tyrosine-2 of hormone analogs with phenylalanine and by nitrating the single tyrosine of the protein. Substitution of phenylalanine for tyrosine in position 2 of binding peptides led to almost total loss of typical 280-nm ellipticity changes associated with binding, but had little effect elsewhere in the spectrum. Changes in the environment of tyrosine-2 are therefore the major source of the 280-nm ellipticity changes. Nitration of neurophysin had no effect on far-ultraviolet ellipticity changes associated with binding, slightly reduced changes in the 230- to 250-nm region, and led to new ellipticity changes above 300 nm which were attributed to perturbation of the nitrotyrosine. Titration studies indicated that the nitrotyrosine pK was lowered 0.6 pH unit by binding. The effects of binding on the ellipticity and pK of the nitrotyrosine were independent of the nature of the peptide side chain in position 1 and of residues 3–9 when differences in binding affinity were allowed for. These data are interpreted in terms of a model which places the neurophysin tyrosine near the protonated α-amino and the side chain in position 2 of the bound peptide. Of changes elsewhere in the spectrum which accompany interaction of native neurophysin with the hormones, the results further implicate disulfide transitions in ellipticity changes above 291 nm and in the 230- to 250-nm region. Far-ultraviolet changes are tentatively attributed principally to side-chain chromophores.

The specific noncovalent complexes of the neurophysins with oxytocin and vasopressin are of interest not only because of their role in the physiology of the hormones (Sachs, 1969; Sawyer, 1961) but also because they provide useful and accessible models for the study of protein–protein interaction. Studies in this laboratory (Breslow and Abrash, 1966; Bres-

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low *et al.*, 1971) and elsewhere (Stouffer *et al.*, 1963; Ginsburg and Ireland, 1964) suggest that hormone–neurophysin interaction involves binding of the protonated α-amino of the hormone together with side chains at positions 1, 2, and 3 to a neurophysin site which contains a carboxyl and other, mainly unidentified, residues.

Large changes in circular dichroism result from the interaction of bovine neurophysin with oxytocin, vasopressin, and appropriate model tripeptides (Breslow, 1970; Breslow *et al.*, 1971). Of these, small changes above 291 nm have been attributed to neurophysin disulfides while changes at shorter wavelengths have been only loosely attributed to either side-chain or peptide-bond chromophores. We wanted to evaluate more specifically the contribution of the single tyrosine of the hormones and the single neurophysin tyrosine to ellipticity changes below 291 nm and so to further define the role of tyrosine in the interaction. To do so we made use of the facts that the tyrosine in position 2 of binding peptides can be replaced by phenylalanine with no appreciable effect on binding (Breslow and Abrash, 1966) and that the single tyrosine of neurophysin can be mononitrated with no diminution in neurophysin–hormone interaction (Furth and Hope, 1970). Both substitution of tyrosine by phenylalanine and mononitration of tyrosine displace tyrosine absorption bands sufficiently (Wetlaufer, 1962; Jaffe and Orchin, 1962) so that most ellipticity changes arising from perturbations of tyrosine transitions should be markedly altered by these substitutions. Because there are no tryptophan residues in either neurophysin or the hormones, the assessment of the role of tyrosine in circular dichroism changes accompanying neurophysin–hormone interaction also allows a further evaluation of the contribution of disulfide and peptide-bond chromophores.

Materials and Methods

Proteins and Peptides. Bovine neurophysin-II[1] was used for all studies and was prepared as described previously (Breslow *et al.*, 1971). Oxytocin, arginine-vasopressin, and lysine-vasopressin were former gifts from Professor V. du Vigneaud and Dr. W. Cash. L-Methionyl-L-tyrosyl-L-phenylalaninamide, L-alanyl-L-tyrosyl-L-phenylalaninamide, and L-cystinylbis-L-tyrosinamide were the peptides described previously (Breslow *et al.*, 1971). *S*-Methyl-L-cysteinyl-L-phenylalanyl-L-isoleucinamide was obtained from Cyclo Chemical Corp., and was reported by Cyclo to be chromatographically homogeneous in three solvent systems. Amino acid analysis (Spackman *et al.*, 1958) in this department gave a ratio of amino acids in this peptide of 0.87 *S*-methylcysteine:1 phenylalanine:1 isoleucine; control studies indicated that the low yield of *S*-methylcysteine was due to destruction of this amino acid during hydrolysis. L-Methionyl-L-tyrosinamide was obtained from Research Plus Laboratories; amino acid analysis showed that it contained a methionine to tyrosine ratio of 0.96:1. H^+ ion titration studies of the latter two peptides indicated that neither had a free α-carboxyl group. L-Methionyl-L-tyrosine and L-methionyl-L-phenylalanine-glycine were obtained from Cyclo Chemical Corp., and also gave the correct amino acid analyses.

Nitration of neurophysin-II was performed as described elsewhere (Furth and Hope, 1970). Disc electrophoresis of the nitrated protein at pH 9.5 (Breslow *et al.*, 1971) showed a single major component of slightly greater anodic mobility than the unmodified protein, and a minor component (10–15% of the total protein) which migrated as the native protein. Amino acid analysis, however, indicated that all the tyrosine was present as 3-nitrotyrosine; no free tyrosine was found. Thus the minor component probably represented a nitrated side product. No attempt was made to separate the two components.

Methodology. Circular dichroism studies were performed on a Cary 60 spectropolarimeter equipped with a Model 6001 circular dichroism attachment as previously described (Breslow, 1970). Protein concentration was 2 mg/ml in 0.16 M KCl for all studies. Path lengths were 1 cm in the near-uv region and 0.1–0.2 mm in the far-uv regions. Short path-length cells were calibrated using *d*-10-camphorsulfonic acid to obtain the exact path length.

For studies in the presence of peptide or hormone, the neurophysin sample was first prepared and its CD spectrum taken in the absence of peptide; peptide was then dissolved directly into the neurophysin solution and the spectrum retaken. Spectra of peptides alone were taken at the same concentrations and path lengths as those used in the presence of neurophysin. Summation of the spectra of peptide and protein taken alone gave the "theoretical" or "calculated" spectra shown for comparison with the observed spectra of neurophysin in the presence of peptide. Near-uv CD spectra, both in the presence and absence of peptide, were calculated as molar ellipticities (*i.e.*, as (deg cm^2)/dmole) using a molecular weight of 10,040 for NP-II. Far-uv spectra were calculated as residue ellipticities ((deg cm^2)/dmole) using a residue weight for NP-II of 105. One "equivalent" of hormone is defined as 1 mole of hormone/10,000 g of NP-II, in keeping with a monomeric weight of 10,000 for NP-II (Breslow *et al.*, 1971).

Estimates of the affinity of NP-II for different peptides were obtained by circular dichroism titration, principally at pH 6.2. Increasing amounts of peptide were added to a solution of native or nitrated NP-II until no further ellipticity changes, other than those generated by the free peptide, were observed. The amount of peptide bound at any one peptide concentration was calculated from the observed ellipticity change at a given wavelength relative to the change induced by saturating concentrations of the same peptide. The relative affinities for different peptides calculated in this manner were in general agreement with affinities calculated by H^+ ion titration at pH 4.0 (Breslow *et al.*, 1971).

Spectrophotometric titration studies of nitrated neurophysin and its complexes were performed at 25° by continuously changing the pH of a 0.2% protein solution (in 0.16 M KCl) with traces of 1 N NaOH and reading the absorbance at each pH on a Gilford Model 222 A spectrophotometer equipped with a digital absorbance meter. Sufficient buffering was provided by the protein so that no significant pH change occurred between the time at which the pH and the absorbance were measured. (Buffering was undoubtedly aided by the fact that no attempt was made to deaerate the sample, *i.e.*, CO_2 and HCO_3^- in equilibrium with air were present at all pH values.) All miscellaneous reagents were reagent grade and deionized water was used throughout.

Results

Ellipticity Changes Accompanying Neurophysin–Oxytocin Interaction. Circular dichroism studies of hormone–NP in-

[1] Abbreviations used are: NP, bovine neurophysin; NP-II, bovine neurophysin-II; CD, circular dichroism; Met-Tyr-PheNH$_2$, L-methionyl-L-tyrosyl-L-phenylalaninamide; *S*-Me-Cys-Phe-IleNH$_2$, *S*-methyl-L-cysteinyl-L-phenylalanyl-L-isoleucinamide; Met-Phe-Gly, L-methionyl-L-phenylalanylglycine; Met-TyrNH$_2$, L-methionyl-L-tyrosinamide; Met-Tyr, L-methionyl-L-tyrosine.

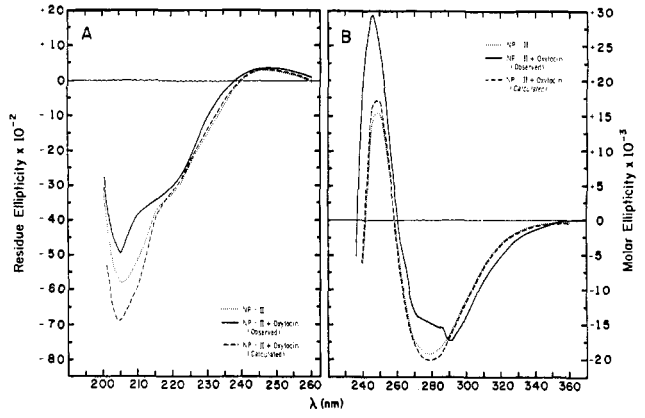

FIGURE 1: Circular dichroism spectra of NP-II in the absence and presence of 1.4 equiv of oxytocin; pH 6.2, 0.16 M KCl. (. . .) NP-II, (——) NP-II + oxytocin (observed spectra), and (– – –) NP-II + oxytocin (calculated spectra). "Calculated" spectra represent the theoretical sum of a mixture of NP-II and 1.4 equiv of oxytocin in the absence of interaction. NP-II is 90% saturated with oxytocin at the levels of oxytocin added.

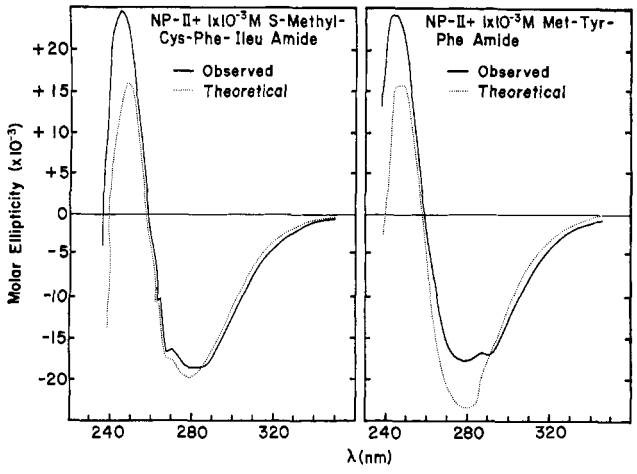

FIGURE 2: Near-uv CD spectra of NP-II in presence of *S*-Me-Cys-Phe-IleNH$_2$ (left) and Met-Tyr-PheNH$_2$ (right). "Theoretical" curves represent calculated spectra for a solution of NP-II which is 1×10^{-3} M in the appropriate peptide in the absence of interaction.

teraction are best carried out at pH 6.2 since binding diminishes with increase in pH and the hormone–NP complex is insoluble between pH 3.5 and 6.2. Figure 1 shows the circular dichroism pattern of native NP-II in the absence of oxytocin and in the presence of excess oxytocin; the observed curves in the presence of oxytocin are compared to calculated (theoretical) curves representing the sum of the individual contributions of the free neurophysin and of free oxytocin obtained separately. The near-uv CD spectrum of free NP-II in Figure 1 is the same as that reported previously (Breslow, 1970; Breslow *et al.*, 1971); the negative ellipticity near 208 nm however is closer to a residue value of −5400 (deg cm^2)/dmole calculated from original data (Breslow, 1970) than to a value of −4500 reported subsequently (Breslow *et al.*, 1971). These data allow a slightly greater α-helix content than the 2% previously calculated. It is also noteworthy that Figure 1 reveals a small negative shoulder at 225 nm more clearly than previously shown (Breslow *et al.*, 1971).[2]

The observed ellipticity spectra of neurophysin in the presence of oxytocin deviate significantly from the calculated spectra for a noninteracting mixture of protein and hormone in four discrete wavelength regions (Figure 1). A slightly increased negative ellipticity occurs above 291 nm and has been ascribed chiefly to changes in neurophysin disulfides (Breslow, 1970). Other changes are increases in positive ellipticity and are roughly centered at 280, 235, and 205 nm, the increase in positive ellipticity of the 248-nm ellipticity band apparently being associated with the 235-nm change (Breslow, 1970). Ellipticity changes generated by binding of vasopressin are similar to those generated by oxytocin binding and indicate that a change in residue three (from isoleucine to phenylalanine) is without significant effect on ellipticity changes accompanying binding.

Ellipticity Changes Occurring on Binding Peptides Substituted with Phenylalanine in Position 2. The binding to neurophysin of tripeptide analogs of oxytocin and vasopressin, such as *S*-Me-Cys-Tyr-PheNH$_2$ or Met-Tyr-PheNH$_2$ is accompanied by the same near-uv ellipticity changes as those induced by hormone (Breslow, 1970; Breslow *et al.*, 1971). In Figure 2, the near-uv effects of binding Met-Tyr-PheNH$_2$ are compared to those of binding *S*-Me-Cys-Phe-IleNH$_2$. (From studies cited above, it is clear that the only significant difference in these peptides is the substitution of phenylalanine for tyrosine in position 2.) Essentially identical ellipticity changes in the 235- to 250-nm region occur on binding of both peptides indicating that changes in this region are not due to the tyrosine of the peptide. However, the typical ellipticity changes near 280 nm associated with binding of hormone or peptides such as Met-Tyr-PheNH$_2$ are almost totally absent when *S*-Me-Cys-Phe-IleNH$_2$ is bound. Binding of both peptides in Figure 2 can be shown to have proceeded to the same extent and to be about 85% complete in both instances; thus the difference at 280 nm between the peptides is not due to weaker binding of *S*-Me-Cys-Phe-IleNH$_2$. Nor is the difference at 280 nm between peptides ascribable to differences in positions 1 and 3 since, as cited above, *S*-Me-Cys-Tyr-PheNH$_2$, Met-Tyr-PheNH$_2$, oxytocin, and vasopressin all generate similar 280-nm changes on binding (Breslow, 1970; Breslow *et al.*, 1971); we have also observed that Met-Phe-Gly produces the same ellipticity changes as does *S*-Me-Cys-Phe-IleNH$_2$ at equivalent levels of binding. Finally, it can be pretty well excluded that the effect of substituting phenylalanine for tyrosine in position 2 is an indirect one resulting from a change in perturbation of some protein chromophore at the binding site when the OH of the tyrosine is lost. The only potential protein chromophores absorbing at 280 nm which could be differentially perturbed by phenylalanine relative to tyrosine are the single tyrosine and the disulfides. Nitration studies (see below) indicate that the single protein tyrosine is identically perturbed by peptides containing tyrosine in position 2 and those containing phenylalanine in position 2. A differential effect on protein disulfides by tyrosine and phenylalanine seems unlikely because both peptides produce the same effect on disulfide ellipticity about 291 nm (see Figure 2). The explanation of the 280-nm differences generated by the two peptides therefore is that the increase in positive ellipticity which accompanies binding of peptides containing tyrosine in position 2 is due principally to changes in the environment of that tyrosine.

[2] A rigorous analysis of the far ultraviolet ellipticity data in terms of per cent β structure and random coil has not been done. Although it was suggested previously that side-chain contributions might dominate the far-uv NP-II ellipticity spectra (Breslow *et al.*, 1971), it is relevant that the data are not incompatible with a significant β-structure content.

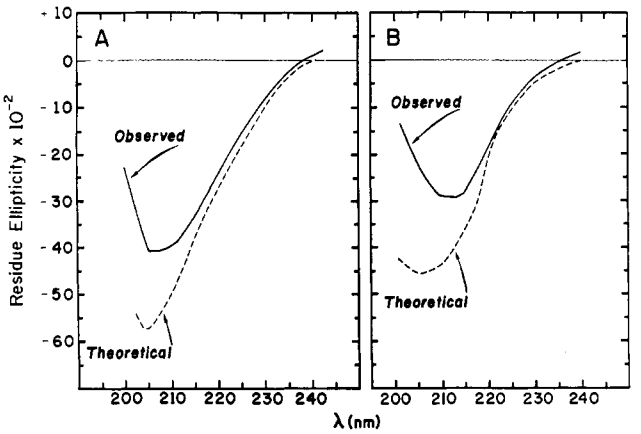

FIGURE 3: Far-uv CD spectra of NP-II in presence of 1×10^{-3} M *S*-Me-Cys-Phe-IleNH$_2$ (A) and in presence of Met-Tyr-PheNH$_2$ (B). Theoretical curves were calculated as in Figures 1 and 2. Marked differences between the two theoretical curves reflect marked far-uv differences between the two peptides.

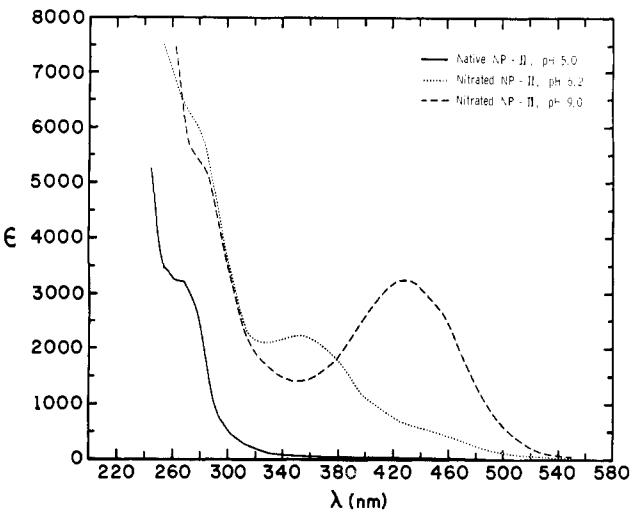

FIGURE 4: Absorption spectra of native and nitrated NP-II. The ordinate is the molar absorptivity.

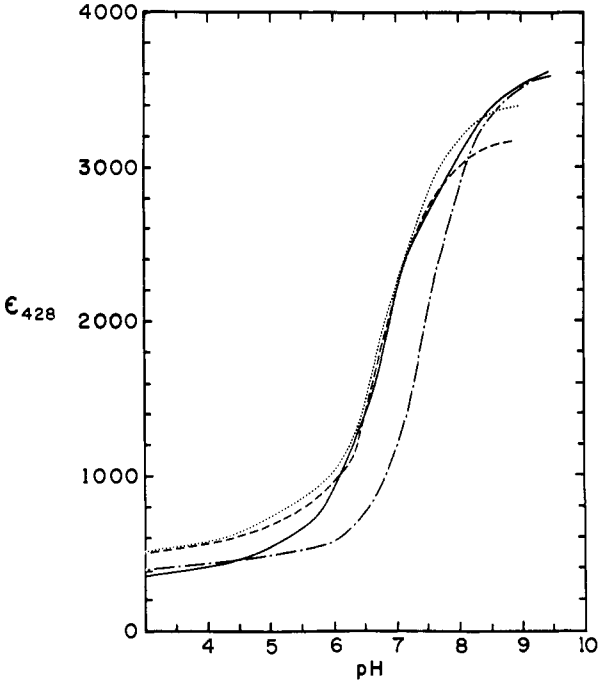

FIGURE 5: Spectrophotometric titration of nitrotyrosine in nitrated NP-II in presence and absence of added peptides plotted as the change in molar absorptivity *vs.* pH at 428 nm. (·····) No peptide; (——) in presence of 10^{-3} M *S*-Me-Cys-Phe-IleNH$_2$; (– – –) in presence of excess (1.4 equiv) oxytocin; and (. . . .) in presence of excess (1.6 equiv) arginine-vasopressin. NP-II is 90% saturated with hormone and 85% saturated with peptide where these are present. Small differences in values of ϵ_{428} at the extremes of pH, between samples containing hormone and those which do not, probably reflect effects of hormone binding and do not significantly affect apparent p*K* estimates (obtained from the midpoint of each curve). The titration curve obtained in the absence of peptide or hormone shows no significant deviation from a theoretical titration curve calculated for a monovalent acid with pK = 7.45. Titration curves in the presence of lysine-vasopressin are not shown but are identical with those obtained in the presence of arginine-vasopressin.

In Figure 3, ellipticity changes occurring in the far-uv region upon binding to neurophysin of *S*-Me-Cys-Phe-IleNH$_2$ and Met-Tyr-PheNH$_2$ are compared. Spectra in this region are less accurate than those in the near-uv region because of the low signal to noise ratio which, in these studies, is compounded by the presence of relatively high peptide concentrations; small differences in the far-uv difference spectra generated by binding of the two peptides therefore cannot be accurately assessed. On the whole, however, the data show that both peptides cause a decrease in negative ellipticity at 205 nm of comparable magnitude to that generated by binding of the hormones, and suggest that the change in negative ellipticity at 205 nm upon hormone binding is not solely attributable to perturbation of the hormone tyrosine in position 2.

Properties of Nitrated Neurophysin. Figure 4 shows the absorption spectra of mononitrated NP-II and native NP-II. The 352-nm band seen in nitrated NP-II at pH 6.2 represents the shifted $^1A_{1g} \rightarrow {}^1B_{2u}$ transition of the protonated nitrotyrosine which, in unmodified tyrosine, occurs near 275 nm (Meloun *et al.*, 1968). Upon ionization (pH 9 spectrum) this band shifts to 430 nm. Differences in absorption between native and nitrated NP-II also occur at shorter wavelengths and are attributable to shifts in other aromatic ring transitions upon nitration. The shoulder seen near 280 nm in the nitrated protein represents the shifted $^1A_{1g} \rightarrow {}^1B_{1u}$ transition of the aromatic ring (Meloun *et al.*, 1968; Jaffe and Orchin, 1962) which, in protonated unmodified tyrosine, occurs near 225 nm (Jaffe and Orchin, 1962; Wetlaufer, 1962). The intensities of the 352- and 430-nm bands are in excellent agreement with those found for mononitrated derivatives of trypsin inhibitor (Meloun *et al.*, 1968). The apparent p*K* of the nitrated neurophysin tyrosine (determined spectrophotometrically as shown in Figure 5) is 7.45 in general agreement with that determined previously (Furth and Hope, 1970); it can be compared to a value of 6.9 found for *N*-acetyl-3-nitrotyrosinamide under similar ionic conditions (Cuatrecasas *et al.*, 1968).

Figure 6 shows the CD spectra of mononitrated NP-II at pH 6.2 and 9 and the spectrum of native NP-II at pH 6.2. At pH 6.2, the 248- and 280-nm ellipticity bands of native NP-II are unaltered by nitration, in keeping with the assignment of these bands predominantly to disulfide transitions (Breslow, 1970). These data also indicate that the 280-nm transitions of the protonated nitrotyrosine have little optical activity under these conditions. However, the 352-nm transi-

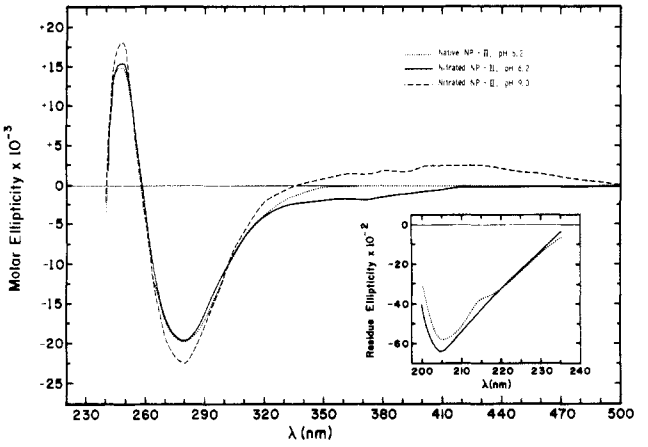

FIGURE 6: Comparison of circular dichroism spectra of native and nitrated NP-II in 0.16 M KCl. (. . . .) Native NP-II, pH 6.2; (——), nitrated NP-II, pH 6.2; (– – – –) nitrated NP-II, pH 9.0.

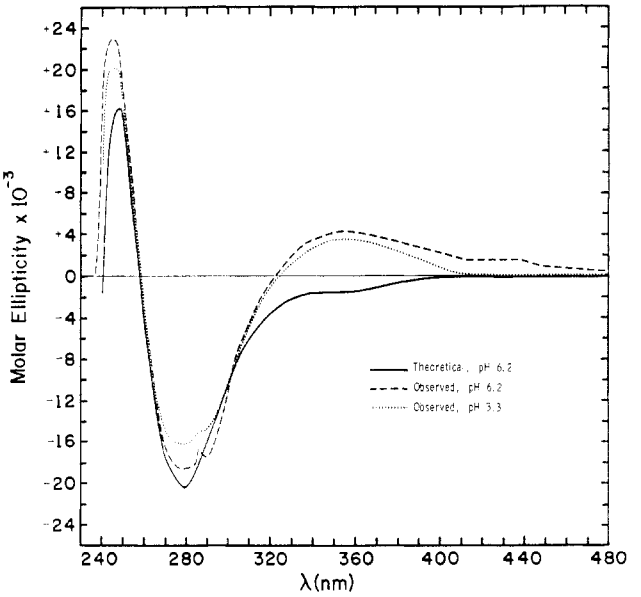

FIGURE 7: Visible and near-uv CD spectra of nitrated NP-II in the presence of 0.7 equiv of oxytocin. Theoretical spectra represent calculated sum for a noninteracting mixture of NP-II and 0.7 equiv of oxytocin.

tion of the protonated nitrotyrosine is weakly optically active, as indicated by a band of negative ellipticity centered near 350 nm at pH 6.2. On raising the pH to 9, a broad positive ellipticity band is generated at 430 nm and can be assigned to the ionized nitrotyrosine residue. In addition, the negative 350-nm band seen at pH 6.2 is replaced by a weak positive ellipticity near 350 nm at pH 9; it is not clear whether or not this represents a tail of the 430-nm transition. Finally, although the near-uv CD spectrum of native NP-II is constant between pH 6.2 and 11 (Breslow, 1970), raising the pH of nitrated NP-II to 9 increases the intensity of both the 248- and 280-nm transitions. The 280-nm increase can probably be assigned to an increase in optical activity of the 280-nm nitrotyrosine transition attendant to ionization, but the nature of the 248-nm effect is unclear.

Upon lowering the pH of uncomplexed nitrated NP-II from 6.2 to 3 (data not shown), the negative 350-nm ellipticity band is replaced by a weaker positive band: the midpoint of this change appears to be between pH 4.5 and 5 and small ellipticity changes are also observed between 285 and 310 nm. We have also observed that similar changes in the 285- to 310-nm region occur with native NP-II at pH 4.5 and are associated with changes elsewhere in the spectrum, particularly in the far-uv region. The data indicate that a change (probably of conformation) occurs in native and nitrated NP-II at low pH; this change is reversible, since all preparations examined at pH 6.2 have been prepared by initial solution at pH 3 (Breslow, 1970). It is possible that the pH 4.5 change is due to titration of a specific carboxyl group, since it occurs in a region of low net charge (bovine NP-II has an isoionic point of 4.95).

Nitration has only small effect on neurophysin secondary structure, as judged by circular dichroism. The inset in Figure 6 compares the far-uv CD spectra of a sample of native and of nitrated NP-II obtained under identical conditions. This particular study was selected for illustration because it represents the maximum difference we have observed to date in the far-uv spectra of the two proteins; in general these spectra of the two proteins are virtually superimposable.

Interaction of Nitrated NP-II with Hormones. Figure 7 shows the ellipticity spectrum of nitrated NP in the presence of 0.7 equiv of oxytocin. At pH 6.2 the binding of oxytocin leads to a conversion of the negative 350-nm nitrotyrosine band to a strong positive band and to the appearance of weak positive ellipticity above 400 nm. The data clearly indicate that the nitrotyrosine is perturbed when hormone is bound.

Of the ellipticity changes which accompany oxytocin binding to native NP-II, changes in the 248- and 280-nm regions also occur in the nitrated protein although, in particular, the magnitude of the 280-nm change is reduced in the nitrated protein at pH 6.2. This effect, together with the positive ellipticity occurring above 400 nm, can be shown to be due to an increased ionization of the nitrotyrosine in the complex at pH 6.2 (see also, below). At pH 3.3 (at which it can be demonstrated that most of the oxytocin is still bound but the nitrotyrosine of the complex is protonated), the ellipticity band above 400 nm disappears and the ellipticity of the complex at 280 nm becomes more positive, leading to an ellipticity pattern at 280 nm which is more similar to that seen with the complex of the native protein (Figure 7). Similarly (Figure 8), an increase in pH of the nitrated NP–oxytocin complex from 6.2 to 7 leads to an increase in positive ellipticity above 400 nm and an increase in negative ellipticity near 280 nm. The magnitude of the 430- and 280-nm ellipticities of the complex at pH 7 indicate that the ionized nitrotyrosine of the complex is more optically active than the ionized nitrotyrosine of the free protein.

The apparent partial ionization of the nitrotyrosine of the complex at pH 6.2 suggested that the pK of the nitrotyrosine of the hormone complex was lower than that of the uncomplexed nitrated protein. This was confirmed by spectrophotometric titration studies (Figure 5) which indicated that, in the presence of oxytocin, vasopressin, or smaller peptides such as *S*-Me-Cys-Phe-IleNH$_2$, the pK of the nitrotyrosine is lowered from 7.45 to 6.85. (It can be shown by CD that no appreciable dissociation of these complexes occurs below pH 6.85 so that the pK measured at the midpoint is valid. Gradual dissociation with pH does occur above pH 7.5, however.)

Figure 8 also shows the far-uv changes accompanying oxytocin binding to the nitrated protein. Comparison of these data to those for the native protein under identical conditions (Figure 1) indicates that the magnitude and direction of the

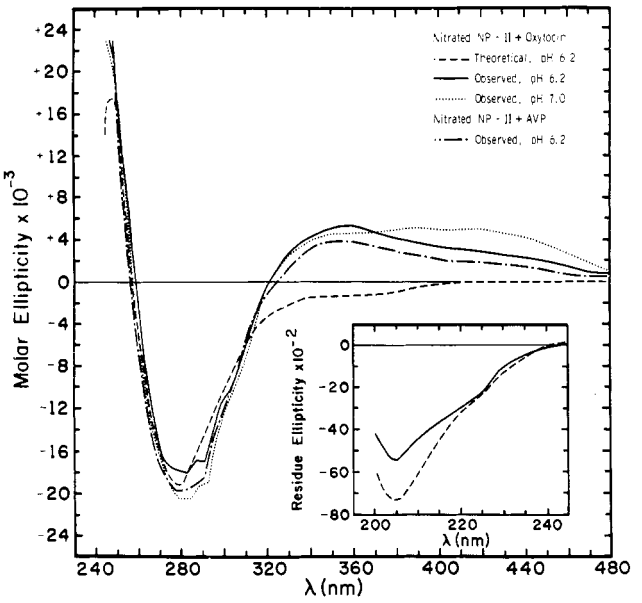

FIGURE 8: Circular dichroism spectra of nitrated NP-II in the presence of excess oxytocin (1.4 equiv) and in the presence of excess arginine-vasopressin (1.6 equiv). "Theoretical" spectra are calculated for oxytocin case only and represent expected spectrum of noninteracting mixture. Symbols in inset have the same significance as in main figure. Excessive noise in these runs presented spectra from being obtained below 245 nm in presence of hormone.

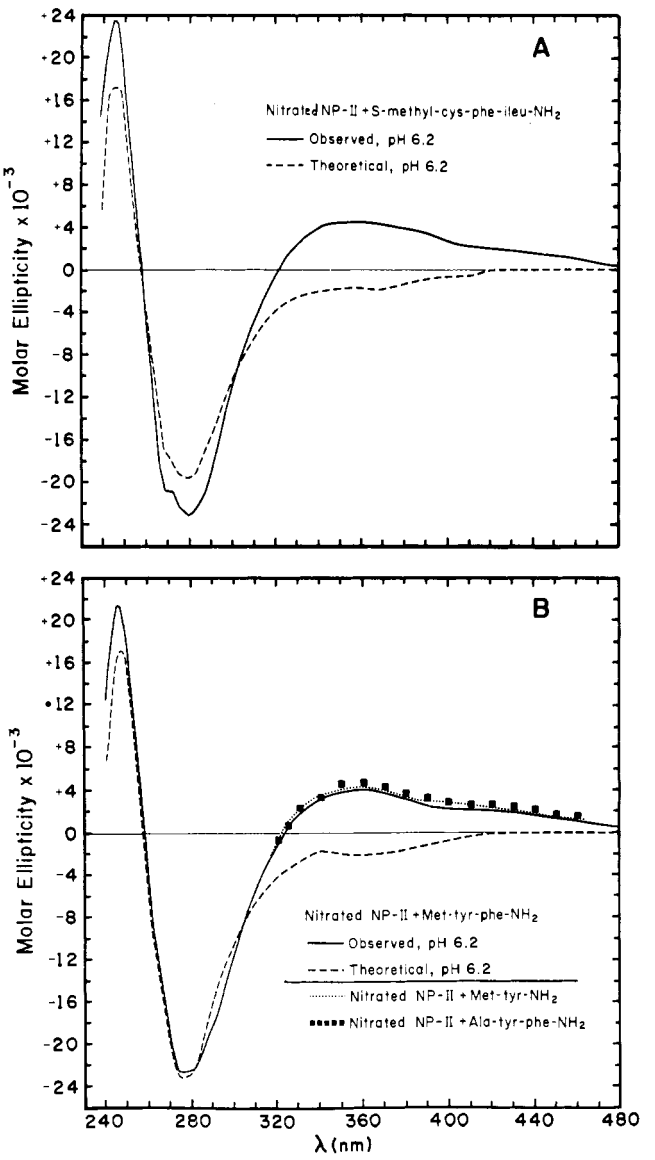

FIGURE 9: Representative circular dichroism spectra of nitrated NP-II in presence of 1×10^{-3} M *S*-Me-Cys-Phe-IleNH$_2$ (A) and 1×10^{-3} M Met-Tyr-PheNH$_2$ (B). Theoretical spectra were calculated as in Figure 2. Also shown in (B) are segments of observed spectra in presence of 2×10^{-3} M Met-TyrNH$_2$ and 4×10^{-3} M Ala-Tyr-PheNH$_2$. Nitrated NP-II is 80–85% saturated with each peptide under the conditions shown.

far-uv ellipticity changes accompanying binding are unaffected by nitration.

Finally, we have studied the effect of vasopressin upon the CD spectrum of nitrated neurophysin. Figure 8 shows the ellipticity changes accompanying binding of arginine-vasopressin to nitrated NP-II. Although ellipticity changes above 300 and near 280 nm are quantitatively slightly lower than those obtained with oxytocin, they are qualitatively identical and can be shown to respond to changes in pH in an identical manner as do those of oxytocin complexes. Lysine-vasopressin (not shown) also affected the CD spectrum of nitrated NP-II in a qualitatively identical manner as did oxytocin except that, in this case, the magnitude of the ellipticity changes were about 10% greater than that seen with oxytocin. Changes in nitrotyrosine pK induced by binding lysine-vasopressin were quantitatively identical with those induced by arginine-vasopressin and oxytocin. As before (Breslow, 1970) small quantitative ellipticity differences produced by different hormone preparations are tenatively attributed to traces of dimer or other hormone aggregates.

Interaction of Nitrated NP-II with Small Peptides. The interaction of nitrated NP-II with several dipeptides and tripeptides was studied by circular dichroism and, in some cases, by spectrophotometric titration. Peptides studied were Met-Tyr-PheNH$_2$, *S*-Me-Cys-Phe-IleNH$_2$, Ala-Tyr-Phe-NH$_2$, Met-TyrNH$_2$, Met-Phe-Gly, Met-Tyr, and L-cystinylbis-L-tyrosinamide. Binding constants for all peptides were determined by circular dichroism and will be reported in a separate communication. When differences in binding affinity were allowed for, all peptides produced the same effects on the ellipticity of the neurophysin nitrotyrosine residue. Selected examples are shown in Figure 9. At 1×10^{-3} M, Met-Tyr-PheNH$_2$ and *S*-Me-Cys-Phe-IleNH$_2$, which have comparable binding affinities and which almost saturate NP-II (and the nitroderivative) at this concentration, produce essentially identical ellipticity effects above 300 nm; similar effects are produced by Ala-Tyr-PheNH$_2$ and Met-TyrNH$_2$ although higher concentrations are necessary because of the weaker binding of these peptides (Breslow *et al.*, 1971). It should be noted that apparent small qualitative differences in Figure 9 among these peptides above 300 nm are well within experimental error and are not reproduced in different experiments. The effects on nitrotyrosine ellipticity produced by binding of these peptides are also, within the limits of experimental error, identical with those induced by hormone binding (compare Figures 8 and 9). The similar effects of small peptide binding on the nitrotyrosine environment is also seen in Figure 5 where it is evident that the change in nitrotyrosine pK induced by binding *S*-Me-Cys-Phe-IleNH$_2$ is identical with that induced by the hormones. A similar pK change was induced by Met-TyrNH$_2$; interestingly, Met-Phe-Gly- and

Met-Tyr, both of which carry a more negative charge than the other peptides,[3] also cause similar changes in ellipticity and nitrotyrosine pK when binding differences are allowed for.

Comparison of the data in Figure 9 to that in Figure 6 shows that, as with binding of oxytocin to nitrated NP-II at pH 6.2, increases in positive ellipticity at 280 nm which accompany binding of Met-Tyr-PheNH$_2$ to native NP-II are lost in the case of the nitrated protein. With S-Me-Cys-Phe-IleNH$_2$, binding to the nitrated protein results in an increased negative ellipticity at 280 nm. Again, the lack of a large positive 280-nm change with Met-Tyr-PheNH$_2$ at pH 6.2 is attributable to the induced negative ellipticity in the 280-nm nitrotyrosine transition of the protein; the induced negative ellipticity of this transition accounts as well for the observed negative change at 280 nm on binding S-Me-Cys-Phe-IleNH$_2$. These results show that even with the nitrated protein, peptides which contain a tyrosine in position 2 produce a more positive ellipticity change at 280 nm than those which have a phenylalanine in position 2 and one which is more positive by an amount roughly comparable to that seen with the native protein. Such an effect must mean that the increased positive ellipticity caused by peptides with a tyrosine in position 2 is due to an increased positive ellipticity of that tyrosine and not to its effect on the tyrosine of the protein. This is so because the 280-nm nitrotyrosine transition should not respond in the same way to perturbation as would the 280-nm tyrosine transition of the unmodified protein (they are different transitions), and because the nitrotyrosine transitions above 300 nm respond similarly to perturbation by both Met-Tyr-PheNH$_2$ and S-Me-Cys-Phe-IleNH$_2$ (Figure 9).

Figure 9 also shows that binding of both peptides to nitrated NP-II leads to an increase in positive ellipticity at and below 248 nm, although the magnitude of the the effect is about 30% lower than with the native protein (Figure 2). On the average, we have observed that binding of the hormones (compare Figures 1, 7, and 8) and of most peptides to nitrated NP-II is accompanied by somewhat lesser ellipticity changes at 248 nm than those accompanying binding to the native protein.

Discussion

The effect of replacing the tyrosine in position 2 of the binding peptide by phenylalanine shows clearly that most of the ellipticity change accompanying binding between 265 and 285 nm is due to perturbation of the tyrosine in position 2 of the peptide. From Figures 1, 2, and 9, the 275-nm molar ellipticity change in this tyrosine on binding can be estimated as approximately +4000 (deg cm^2)/dmole. Since the molar ellipticity of small tyrosine derivatives in this wavelength region is typically about ±1000 (deg cm^2)/dmole (Beychok, 1968), and was actually about −500 (deg cm^2)/dmole in our tyrosine-containing peptides when unbound, this large change suggests a high degree of assymmetric orientation of tyrosine-2 in the complexed state. Although no comparable change in the phenylalanine region of the spectrum is seen upon binding peptides containing a phenylalanine in position 2, such an effect could well go unnoticed, both because it would be expected to be considerably weaker and also because the wavelength region in which phenylalanine absorption is found (240–270 nm) is on the steep edges of two large ellipticity bands. In this respect, it is relevant to point out that the small ellipticity changes between 270 and 285 nm noted on mixing peptides with a phenylalanine in position 2 and native NP-II (Figure 2) cannot (by virtue of their position) be generated by phenylalanine and are probably due to perturbation of the tyrosine of the protein.

The major contribution of tyrosine-2 of the binding peptide to ellipticity changes near 280 nm parallels the highly important role this tyrosine plays in binding. Thus, it was earlier shown, that the binding site on the protein has a high degree of specificity for a tyrosine or phenylalanine in position 2 (Breslow and Abrash, 1966) and will not accept an isoleucine or glycine, although isoleucine analogs of the hormones have considerable hormonal activity. The binding data and the present results both support a highly constrained position for the peptide aromatic ring of position 2 in the peptide–protein complex. It is of interest here that tyrosine-2 of arginine-vasopressin contributes to a spectrophotometric difference spectrum generated by binding of hormone to NP-II (Furth and Hope, 1970).

Perturbation of the nitrotyrosine of the nitrated protein by binding is in accord with spectrophotometric studies (Furth and Hope, 1970) which indicated that the nitrotyrosine absorption spectrum was perturbed on binding arginine-vasopressin, and which suggested that the pK of the nitrotyrosine might be lowered by binding. Our data indicate that the pK decrease is 0.6 pH unit. Since such an effect can be calculated (Linderstrom-Lang, 1924) to be too great to arise only from the +1 change in net protein charge which accompanies peptide binding, the data instead signify a change in the immediate vicinity of the nitrotyrosine when peptide is bound. It was previously suggested (Furth and Hope, 1970) that the source of the pK decrease was most probably the proximity of the positively charged side chain (lysine or arginine) in position 8 of the vasopressins. Our data indicate that an identical change in pK and perturbation occurs with oxytocin (where position 8 is leucine) as well as with di- and tripeptides. Thus, the nitrotyrosine perturbant is not position 8 of the vasopressins, nor can it be any of the residues in positions 3–9, the side chain in position 1 or the OH of tyrosine-2. It is also relevant to note that, in additional contrast to the conclusions of Furth and Hope (1970), we find nothing in our data which suggests an increase in hydrophobicity of the nitrotyrosine environment when peptide is bound; *i.e.*, the shift in nitrotyrosine ellipticity to longer wavelengths is accountable for solely by the increase in nitrotyrosine ionization which accompanies peptide binding.

The independence of nitrotyrosine perturbation from the nature of the bound peptide might suggest that perturbation is secondary to a conformational change accompanying binding. However, very recent nuclear magnetic resonance (nmr) studies (Balaram *et al.*, 1972) indicate that, in the peptide–protein complex, the neurophysin tyrosine is close to the aromatic ring in position 2 of the peptide. It is of interest to correlate this with our observations here. For example, the proximity of the neurophysin tyrosine to position 2 of the peptide suggests that it may also be somewhat near the carboxyl with which the protonated α-amino of the peptide interacts. The general proximity of the tyrosine to a carboxyl is supported by the titration data. The apparent pK of the nitrotyrosine in free NP-II is 0.55 pH unit higher than that of N-acetyl-3-nitrotyrosinamide (see Results), which we believe to be the appropriate model for a nitrotyrosine in an

[3] It is possible that these peptides bind only in the COOH form (in which case their charge is the same as that of the other peptides) but this appears tentatively unlikely. Met-Phe-Gly binds with an affinity about 1/80th that of Met-Tyr-PheNH$_2$. Everything else being equal, the necessity to protonate the α-COOH should reduce the affinity by a factor of about 500 at pH 6.2.

uncharged environment. Using previous titration data (Breslow *et al.*, 1971) it can be calculated that, at best, only half of this pK displacement can be due to distributed electrostatic repulsions (Linderstrom-Lang, 1924); the high pK of the nitrotyrosine therefore suggests the influence on tyrosine of a negative group which can only be a carboxyl.[4] On the basis of the above, we suggest that the diminution in pK of the nitrotyrosine when peptide binds is due, at least in part, to neutralization of charge on the proximal carboxyl by the protonated α-amino of the peptide. Marked changes in nitrotyrosine ellipticity attendant to binding can be explained as a result of interaction with position 2 of the peptide (and perhaps with the α-amino as well) in such a manner that the two aromatic rings are close, but that the side chains in positions 1 and 3–9 of the peptide, and the OH of tyrosine-2 (all of which can be lost or modified with no effect on nitrotyrosine ellipticity) make no direct contact with the tyrosine of the protein. The lack of contact between position 3 of the peptide and the NP-II tyrosine is also suggested by the nmr studies cited above.

It is of interest in the above context to speculate as to the probable protein carboxyl involved in hormone binding. Titration studies (Breslow *et al.*, 1971) suggest that this is a side-chain carboxyl; this is supported by sequence studies (Capra *et al.*, 1972) which indicate a variability in the carboxyl termini of the different neurophysins. Bovine NP-II contains large regions which are common to other neurophysins, some of which represent internally duplicated segments; it has been suggested previously that the binding site lies within these regions common to all neurophysins (Capra *et al.*, 1972). If one adds the restriction that the active-site carboxyl must lie in a region which is not duplicated within the same polypeptide chain (since there is only one binding site per chain) then the carboxyls involved can be only Glu-40, Glu-46, or Glu-47. Interestingly, all of these carboxyls are relatively close to the tyrosine (position 49) in the sequence, but the validity of the above restriction is admittedly not certain.

Demonstration that the 350-nm protein nitrotyrosine transition is perturbed by binding raises the question as to whether the corresponding transition (275 nm) of the unmodified tyrosine of native NP-II is perturbed. As noted above, positive ellipticity changes seen in the 268- to 285-nm region on binding *S*-Me-Cys-Phe-IleNH$_2$ to native NP-II are probably attributable to changes in protein tyrosine ellipticity. Although the magnitude of these changes (about +1000 (deg cm^2)/dmole at 275 nm) are less than those seen with the 350-nm nitrotyrosine transition, this can be attributed in part to the fact that the 350-nm nitrotyrosine transition is more electrically allowed than the 275-nm tyrosine transition. Another question raised by the nitrotyrosine data is whether the negative ellipticity increase above 291 nm seen with the native protein on binding is due, not to disulfide, but to an increased degree of ionization of the unmodified NP-II tyrosine such that it can now contribute to the ellipticity above 295 nm at pH 6.2. This is not the case. First, it is unlikely that the pK of unmodified tyrosine would be sufficiently lowered from 9.7 such that any ionization would occur at pH 6.2; the change in nitrotyrosine pK induced by binding is only 0.6 pH unit. Secondly, such an increased ionization would be markedly enhanced as the pH of the NP-II complex was increased above 6.2. Studies in this laboratory indicate that the ellipticity of the protein–hormone complex above 291 nm does not change with increase in pH until dissociation of the complex begins. Thus, the ellipticity changes occurring above 291 nm in the native protein remain assigned to protein disulfide.

The present studies also allow a tentative evaluation of the contribution of residues other than tyrosine to binding-induced ellipticity changes in the 230- to 250-nm region. Such changes are seen to be unaffected by substitution of tyrosine-2 in the peptide by phenylalanine and are only partially diminished by nitration; in addition, we have preliminary evidence that the diminution induced by nitration is a variable function of the particular protein preparation and is not intrinsic to the substitution of nitrotyrosine for tyrosine. The 230- to 250-nm changes therefore have their major origin in nontyrosine chromophores, among which only disulfides, phenylalanines, and peptide bonds can absorb in this region. Of these, phenylalanine ellipticity is generally much weaker than the observed changes (which, on a molar basis, are at least 20,000 (deg cm^2)/dmole at 240 nm) and is characterized by fine structure not seen in the 248-nm ellipticity band in the complex (Menendez and Herskovits, 1970). Peptide-bond transitions, including the 222-nmn $\rightarrow \pi^*$ transition which is the important one in this region, are generally assumed not to be significant above 245 nm (Greenfield and Fasman, 1969; Saxena and Wetlaufer, 1971). Moreover, ellipticity changes at 222 nm which accompanying binding are so small (Figures 1, 3, and 8) that is seems unlikely that they can be the origin of changes in the 240- to 250-nm region (see also below). Most of the 230- to 250-nm ellipticity increase therefore, or at least of its long-wavelength edge, appears to arise from disulfides, an assignment which is additionally justified by the disulfide origin of the 248-nm ellipticity band in the uncomplexed protein (Breslow, 1970). We have also observed that, under several different conditions (urea, partial reduction), there is a general parallel between the intensity of negative disulfide ellipticity above 291 nm and of positive disulfide ellipticity near 248 nm (E. Breslow and C. Menendez-Botet, unpublished results). Because peptide binding is accompanied by an increased negative disulfide ellipticity above 291 nm, a concommitant increase in positive disulfide ellipticity near 248 nm is probably to be expected.

Ellipticity changes below 215 nm which accompany binding are particularly difficult to assign. Potential chromophores in this region include peptide bonds and a number of side chains. Again, however, the relatively small ellipticity changes near 222 nm are of interest, since this is a region which should be particularly responsive to changes in peptide-bond transitions accompanying changes in secondary structure (Greenfield and Fasman, 1969), It is always possible that the relative constancy of ellipticity near 222 nm is due to cancellation of 222-nm peptide-bond ellipticity changes by concurrent ellipticity changes of opposite sign. Nonetheless, in the absence of evidence supporting such cancellation, and using peptide-bond ellipticity values of Greenfield and Fasman (1969), the only direct interpretation of our 222-nm data is that any changes in secondary structure are small—too small to allow extensive peptide-bond contributions to ellipticity changes below 215 nm. On this basis we tentatively favor assigning the bulk of changes below 215 nm to side-chain chromophores,

[4] Titration studies of the unmodified tyrosine of native NP-II indicated a relatively normal intrinsic tyrosine pK when distributed electrostatic interactions were corrected for (Breslow *et al.*, 1971). This may mean that there is a minor conformational change at the higher pH at which the unmodified tyrosine titrates—or it may reflect the limitations of the Linderstrom-Lang model (Linderstrom-Lang, 1924) when only a single group is titrating. The nitrotyrosine of nitrated NP-II titrates with no obvious spreading due to electrostatic interactions (see Legend to Figure 5).

among which disulfides are candidates of particular interest. Not only do disulfides have significant ellipticity bands near 200 nm (Coleman and Blout, 1968; Ludescher and Schwyzer, 1971), but changes in NP-II disulfide optical activity elsewhere in the spectrum indicate a change in disulfide environment or geometry on binding which might well be manifest in the far-uv region. Takagi and Ito (1972), in temperature studies of L-cystine, observed that near-uv disulfide ellipticity changes were paralleled by far-uv disulfide changes. Of other potential side-chain chromophores, our studies give little evidence that the tyrosine of either NP-II or the peptide are major contributors to the far-uv ellipticity changes. However, it must also be recognized that failure of substitution of phenylalanine for tyrosine in the binding peptide to produce a large effect on far-uv changes may be due to the fact that differences between phenylalanine and tyrosine transitions in the far-uv region are small (Wetlaufer, 1962) and may be within the noise level at 205 nm; an appreciable far-uv contribution by the peptide tyrosine cannot be precluded.

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