Complex Formation between Bovine Neurophysin-I and Oxytocin, Vasopressin, and Tripeptide Analogs of Their NH₂-terminal Region

ABSORBANCE AND CIRCULAR DICHROISM DIFFERENCE SPECTROSCOPIC STUDIES*

(Received for publication, June 11, 1973)

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SUMMARY

Complex formation between bovine neurophysin-I and oxytocin, (S-lysine)vasopressin, or tripeptide analogs of the NH₂-terminal region of these hormones, Cys(Me)-Phe-Ile-NH₂ and Cys(Me)-Tyr-Phe-NH₂, has been studied using ultraviolet absorption and circular dichroism difference spectroscopy between 250 nm and 350 nm. The results suggest that upon complex formation residues 2 and 3 of the hormone molecule are bound in a hydrophobic region of neurophysin, while tyrosine-49 of the neurophysin molecule moves from a hydrophobic to a hydrophilic environment.

Tyrosyl circular dichroism spectral changes due to complex formation have been compared to the corresponding tyrosyl absorption spectral changes. This comparison shows that the vibronic fine structure in tyrosyl circular dichroism difference spectra may be used to characterize the microenvironment of tyrosyl residues in proteins.

Oxytocin and vasopressin are peptide hormones which are stored prior to their secretion in the mammalian posterior pituitary gland. These hormones are found complexed with a class of proteins, the neurophysins, in neurosecretory granules in the pituitary (1-3). Studies of the isolated neurophysin-hormone complexes may contribute to our understanding of the structure of the hormone as it exists in the neurosecretory granules. Moreover, the complexes formed between isolated neurophysin molecules and the neurohypophysial hormones provide a model system for the study of the binding of peptide hormones by proteins. Additionally, since the neurophysin molecules contain only one tyrosine residue and no tryptophan residues, these hormone-protein complexes are favorable for studying the circular dichroism (CD) properties of tyrosyl chromophores in proteins.

Spectroscopic studies of nitrated neurophysin led to the suggestion that the binding of hormone by neurophysin is accompanied by perturbations of the single tyrosyl residue of neurophysin (4-6). The involvement of the single tyrosyl residue of the hormone has also been proposed on the basis of indirect binding studies using native and chemically modified peptides (7-9). Further support for this hypothesis has come from high-resolution proton nuclear magnetic resonance studies using oxytocin (10) and (S-lysine) vasopressin,¹ optical spectroscopic studies using (S-arginine) vasopressin (4), and nuclear magnetic resonance and CD² spectroscopic studies using tripeptide analogs of the NH₂-terminal portion of the hormones (5, 12).

We present here CD and absorbance difference spectra which arise from the binding of oxytocin, (S-lysine)vasopressin, and tripeptides to bovine neurophysin-I. These results are interpreted to define the individual microenvironments of tyrosine-49 of neurophysin-I and of tyrosine-2 and phenylalanine-3 of the hormones in the neurophysin-hormone complex. Additionally, the results provide a clear correlation between tyrosyl absorbance difference spectra and the fine structure in tyrosyl CD difference spectra.

MATERIALS AND METHODS

Highly purified bovine neurophysin-I was obtained by isoelectric focusing, as described elsewhere (9). The synthetic oxytocin and (S-lysine)vasopressin peptides were generously provided by Dr. Guttmann (Sandoz, Basle). The synthetic tripeptides, Cys(Me)-Phe-Ile-NH₂ and Cys(Me)-Tyr-Phe-NH₂, were obtained from Bachem, and used without further purification. Concentrations of neurophysin-I, oxytocin, vasopressin, and tripeptides were determined by dry weight as well as by ultraviolet absorbance measurement, as previously described (9).

Absorbance and CD difference spectra were measured as pre-

¹ R. Alazard, P. Cohen, J. H. Griffin, and J. Cohen, manuscript in preparation.
² The abbreviation used is, CD, circular dichroism.
RESULTS

The absorbance difference spectra between 250 nm and 310 nm due to the binding of either oxytocin or (8-lysine)vasopressin to bovine neurophysin-I are shown in Fig. 1A. The positive absorbance differences at 287 nm and 280 nm with approximate \( A_e \) values of 400 and 170 \( \text{M}^{-1} \text{cm}^{-1} \), respectively, are characteristic of tyrosyl transitions which are red-shifted. Such perturbations are seen when tyrosyl chromophores are rendered inaccessible to aqueous solvent (14). Additional fine structure is apparent between 250 nm and 270 nm in the absorbance difference spectrum for the binding of (8-lysine)vasopressin. This fine structure with a variation of \( A_e \) between 265 nm and 262 nm of 14 \( \text{M}^{-1} \text{cm}^{-1} \) is characteristic of phenylalanine chromophores which are red-shifted and rendered inaccessible to aqueous solvent (14). (8-Lysine)vasopressin contains one phenylalanine residue, while oxytocin has none and only (8-lysine)vasopressin binding gives the absorbance difference spectral characteristics of a red-shifted phenylalanine chromophore. This result suggests that the phenylalanine-3 residue of vasopressin is rendered less accessible to aqueous solvent upon interaction with neurophysin-I.

The binding to neurophysin-I of tripeptide analogs of the NH$_2$-terminal region of the neurohypophysial hormones, such as Cys(Me)-Tyr-Phe-NH$_2$ and Cys(Me)-Phe-Ile-NH$_2$, gives rise to the absorbance difference spectra shown in Fig. 1B. The tyrosyl perturbations for the binding of the tripeptide analog containing a tyrosine residue (Fig. 1B) are very similar to the results seen for hormone binding (Fig. 1A), except that the wave length maxima are shifted 1 nm toward the far ultraviolet. However, for the binding of the tripeptide analog containing phenylalanine in place of tyrosine, the tyrosyl perturbations recorded in Fig. 1B are quite different and are characteristic of a tyrosyl chromophore which is blue-shifted due to an increase in polarity of its microenvironment (14). This tyrosyl perturbation can come only from tyrosine 49 of neurophysin-I, since it is the only tyrosyl residue in the complex (15). Additionally, the binding of this tripeptide gives rise to sharp fine structure between 250 nm and 270 nm, with a variation of \( A_e \) from 265 nm to 262 nm of 100 \( \text{M}^{-1} \text{cm}^{-1} \). This suggests that the phenylalanine chromophore of Cys(Me)-Phe-Ile-NH$_2$ becomes quite inaccessible to aqueous solvent in a hydrophobic environment in the tripeptide-neurophysin-I complex (14).

The CD difference spectra between 250 nm and 330 nm due to the binding of either oxytocin or (8-lysine)vasopressin to neurophysin-I are shown in Fig. 2A. The positive maximum at 279 nm in the difference spectrum is 5500 deg cm$^2$ per dnmole for the binding of either oxytocin binding but 3400 deg cm$^2$ per dnmole for the binding of (8-lysine)vasopressin to neurophysin-I. In addition to the maximum at 279 nm in Fig. 2A, shoulders are seen in the CD difference spectra near 286 nm, 272 nm, and 267 nm. These resolved CD bands characteristically arise from the excitation of different vibronic states in model tyrosyl chromophores in nonaqueous solution (16, 17). Thus, tyrosyl residues in a nonpolar environment contribute significantly to the difference CD spectra be-

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**Fig. 1.** Absorbance difference spectra due to the binding of neurohypophysial hormones (A) and of tripeptide analogs (B) of NH$_2$-terminal region of the hormones to bovine neurophysin-I. The spectral bandwidth was significantly less than 1 nm at all wave lengths. The final concentration of neurophysin I was 0.14 mm and the final concentration of hormone or tripeptide was 1.1 mm in each experiment.

**Fig. 2.** Circular dichroism difference spectra due to the binding of neurohypophysial hormones (A) and of tripeptide analogs (B) of NH$_2$-terminal region of the hormones to bovine neurophysin-I. The spectral bandwidth was less than 1 nm at wave lengths above 260 nm. The root-mean-square of noise was less than 400 deg cm$^2$ per dnmole at all wave lengths. These CD difference spectra were determined on the same samples in the same cuvettes as used to obtain the absorbance difference spectra in Fig. 1.
between 260 nm and 300 nm. However, the broad negative CD band above 290 nm must be assigned to disulfide chromophores in the neurophysin-hormone complex, since unionized tyrosyl chromophores do not possess transitions above 290 nm (6, 16, 17).

The CD difference spectra between 250 nm and 330 nm due to the binding of two tripeptides to neurophysin-I are given in Fig. 2B. The result for the binding of the tripeptide analog containing a tyrosyl residue is very similar to that seen for the binding of hormone in which tyrosyl perturbations are a major component of the difference spectrum. As observed in the absorption difference spectrum, the tyrosyl perturbations due to the binding of Cys(Me)-Tyr-Phe-NH₂ to neurophysin-I are slightly shifted to lower wave lengths in comparison to the results for the binding of the hormones. For this peptide the maximum change in ellipticity of 5900 deg cm² per dmole occurs at 278 nm and there is a shoulder near 285 nm. However, for the binding of Cys(Me)-Phe-Ile-NH₂ to neurophysin-I, the CD difference spectrum shows a much weaker tyrosyl perturbation with the maximum ellipticity change of 1300 deg cm² per dmole at 275 nm and with a shoulder near 282 nm. This tyrosyl perturbation can come only from tyrosine-49 of neurophysin-I. The location of CD bands at 275 nm and 282 nm is seen in the CD spectrum of model tyrosyl chromophores in aqueous solution (16, 17). Thus, as suggested by the absorbance difference data, the CD difference data imply that tyrosine-49 of the neurophysin molecule is more exposed to aqueous solution upon peptide binding. The broad negative CD bands above 290 nm seen in Fig. 2A for the binding of the hormones are not similarly seen for the binding of the tripeptide analogs.

**DISCUSSION**

**Structure of Neurophysin-hormone Complex—Ultraviolet absorption difference spectroscopy is capable of defining changes in microenvironment of aromatic amino acid chromophores which accompany complex formation between protein and ligand molecules (14). The direct involvement of residue 2 and residue 3 of the neurohypophysial hormones in complex formation with neurophysin has been previously proposed on the basis of binding experiments (7–9) and spectroscopic studies (4, 5, 8, 10, 12). However, none of these previous reports have clarified the microenvironment of these residues which occur when oxytocin or vasopressin is bound to neurophysin. The spectral results reported here suggest that tyrosyl chromophores are rendered inaccessible to aqueous solvent when oxytocin or (8-lysine)vasopressin is bound to bovine neurophysin-I. Since each hormone-neurophysin complex contains two tyrosyl residues, specifically tyrosine-2 of the hormone and tyrosine-49 of the neurophysin (16, 17), tripeptide analogs of the NH₂-terminal region of the hormone are useful to determine the respective contributions of the two different tyrosyl chromophores to the tyrosyl spectral perturbations. Binding to neurophysin-I of the tripeptide analog containing a tyrosine residue, Cys(Me)-Tyr-Phe-NH₂, gives tyrosyl difference spectra similar to those seen for hormone binding. Binding to neurophysin-I of the tripeptide analog containing phenylalanine in place of tyrosine, Cys(Me)-Phe-Ile-NH₂, gives tyrosyl spectral perturbations which are very different from those seen for the binding of the hormones or the tripeptide containing tyrosine. Since this neurophysin-peptide complex which contains the single tyrosine-49 of the protein exhibits a blue-shifted tyrosyl difference spectrum, it is concluded that tyrosine-49 is displaced in a more polar environment upon complex formation. It is reasonable to assume that tyrosine-49 is perturbed similarly when either of the tripeptides or of the hormones is bound to neurophysin-I, since it was previously shown that nitrated tyrosine-49 of neurophysin-II shows the same pKₐ changes and CD perturbations when either of these hormones or of these same model tripeptides is bound (5). On this assumption, it is concluded that the tyrosyl chromophore of each peptide is responsible for the red shift in the tyrosyl absorption spectrum. Thus, tyrosine-2 of the hormone molecule is rendered inaccessible to aqueous solvent upon complex formation with neurophysin-I and is bound in a hydrophobic region of the protein.

Two simple schematic representations of the binding of (8-lysine)vasopressin to neurophysin are shown in Fig. 3. Salient features of complex formation which are pictured in Fig. 3 are: (a) the rigid binding of tyrosine-2 and phenylalanine-3 of the hormone in a hydrophobic region of the protein; (b) the movement of tyrosine-49 of neurophysin from a hydrophobic environment to a more polar environment exposed to aqueous solvent; (c) a buried ion pair between the hormone's NH₂-terminal amino group of cysteine 1 and a carboxyl group of neurophysin; (d) additional unspecified interactions between group Y of the hormone and group X of neurophysin. Experimental evidence for feature a of the model comes from binding data (7–9), spectroscopic studies (4, 5, 8, 10, 12), and the spectral data in Figs. 1 and 2. The residues' rigidity is inferred from nuclear magnetic resonance data (10). Strong support for feature b is derived from the spectral results in Figs. 1 and 2. Feature b is also suggested by the observation that the pKₐ of nitrated tyrosine-49 of neurophysin-II is abnormally low at 7.45 in the absence of bound hormone, whereas it is 6.9 in the presence of bound hormone (5). The pKₐ value of 6.9 is typical of the model compound N-Ac-Tyr(3-NO₂)-NH₂ in aqueous solution (18). Evidence for feature c comes from the pH dependence of hormone binding, which suggests the involvement of two titrating groups with apparent pKₐ values of 4.6 and 6.5 (9). These titrating groups are probably carboxyl and ε-amino moieties. The observation that deamino-analogs of the hormones do not bind to neurophysin also suggest feature c (7, 19). Feature d is depicted to account for the fact that tripeptide analogs of vasopressin containing residues 1 to 3 bind to neurophysin with a somewhat lower affinity than the hormone itself (20). It therefore might be necessary for interactions between groups X and Y to contribute a factor of 10 to 30 to the binding affinity constant. It must be emphasized that the schematic models in Fig. 3 are very simple models for complex formation in which no consideration has been made of neurophysin dimerization, the possibility of multiple binding sites, the exact proximity of tyrosine-49 to tyrosine-2, or the possibility of additional conformational changes in either neurophysin or vasopressin.

**Relation of Absorbance to Circular Dichroism Difference Spectroscopy—**In studying the ultraviolet spectral perturbations of aromatic amino acid chromophores in proteins, it is of interest to compare CD difference spectral results (Fig. 2) to absorbance difference spectra (Fig. 1) (13). Complex formation between neurophysin and oxytocin, (8-lysine)vasopressin, or tripeptide analogs is especially valuable in this regard, since such complexes lack tryptophan, and thus only tyrosyl, disulfide, and phenylalanine and phenylalanine chromophores contribute to the absorbance and CD spectra between 250 nm and 350 nm. Of these chromophores, tyrosyl groups exhibit spectral fine structure between 265 nm and 270 nm (16, 17), phenylalanine groups do so between 250 nm and 270 nm (21, 22), and disulfide groups give no fine structure bands between 250 nm and 350 nm (23). The assignment of absorbance

and CD changes to particular kinds of chromophores can be made based on the wave lengths of the observed spectral changes and on the nature of any observed spectral fine structure. Large tyrosyl perturbations are apparent in both the CD and absorbance data for the binding of ligands to neurophysin-I. Perturbations of the hormonal phenylalanine-3 and of phenylalanine residues of the tripeptide ligands are seen in the absorbance and CD data. On the other hand, the CD results above 290 nm record conformational changes in disulfide chromophores which go undetected in the absorbance difference spectra. The tyrosyl absorbance difference peaks are slightly sharper for oxytocin compared to (8-lysine)vasopressin, suggesting that the tyrosyl group of oxytocin may be bound more rigidly to neurophysin-I than that of (8-lysine)vasopressin. This may be correlated with the nature of any observed spectral fine structure. Large intensity changes of the entire spectrum (13). Such a situation clearly exists in the data presented in Figs. 1 and 2. For example, the absorption difference spectra for the binding of ligands containing a tyrosyl residue to neurophysin-I are dominated by a net displacement of tyrosine-2 of the hormone residue can be established from the data presented here. The results show that when tyrosine-3 of the hormone is placed into a hydrophobic environment in the protein structure, as indicated by a red-shifted absorbance spectrum, the fine structure in the CD difference spectrum exhibits its 0-0 transition near 287 nm; and conversely, when tyrosine-49 of neurophysin is placed into a more hydrophilic environment, as indicated by a blue-shifted absorbance spectrum, the fine structure in the CD difference spectrum exhibits its 0-0 transition near 292 nm. As these results provide the first clear demonstration in a model protein system that tyrosyl (CD) fine structure can be used to characterize the microenvironment of tyrosyl residues in proteins, as previously suggested by Strickland and colleagues (16, 17, 24). Consequently, CD difference spectroscopy is well suited for the definition of conformational changes of aromatic residues in proteins and should be viewed as complementary to ultraviolet absorbance difference spectroscopy.

Acknowledgments—We gratefully acknowledge the helpful support and encouragement of Dr. Pierre Fromageot and Dr. Christian B. Anfinsen.

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