[Chem. Pharm. Bull. 24(1) 36 — 45 (1976)]

UDC 577.159.02 : 547.815.1.04

Photooxidation of Bovine Neurophysin II in the Presence of Rose Bengal[1)]

Hideo Fukuda, Takao Hayakawa, Jirō Kawamura,[2a)] and Yoshio Aizawa[2b)]

Department of Biological Chemistry and Standards, National Institute of Hygienic Sciences[2a)] and Department of Pharmacology, Tokyo College of Pharmacy[2b)]

(Received May 6, 1975)

In order to elucidate the role of some amino acid residues in bovine neurophysin II (NP-II) for its binding ability for oxytocin and vasopressin, NP-II has been photooxidized in the presence of rose bengal and oxygen, and the relationships between the loss of hormones-binding ability of modified protein and the destruction of photosusceptible amino acids were examined.

Photooxidation of NP-II causes rapid oxidation of single methionine residue to corresponding sulphoxide followed by a slow destruction of single tyrosine residue. More prolonged irradiation causes also slight decomposition of cystine residues. The hormones-binding ability of NP-II is almost completely retained even when the methionine residue was completely photooxidized, but is gradually decreased with the progress of the photodegradation of the tyrosine residue. The decrease in binding ability of the photooxidized protein proceeds almost identically for oxytocin and [8-arginine]vasopressin as the ligand. The binding ability is decreased to about 70% of the original when 80% of tyrosine were degraded and to about 30% of the original when the tyrosine was completely photooxidized and about 6% of cystine residues were degraded. The influence of the destruction of tyrosine residue for the loss of hormones-binding ability of protein seemed to be amplified by the subsequent photooxidation of cystine residues since there is no direct correlation between the photodestruction of cystine residues and the decrease in the binding ability. The pH binding profiles of photooxidized NP-II are found to be essentially identical with those of native protein, indicating the non-essential role of phenolic hydroxyl group of tyrosine residue of NP-II in hormones-binding process. O-Acetylation of the tyrosine residue of NP-II with N-acetylimidazole gives no significant effect on the binding ability for oxytocin or [8-arginine]vasopressin.

These findings suggest that the single methionine residue of NP-II has no contribution to the binding process, while the single tyrosine residue, particularly its aromatic ring, of NP-II may participate with the binding process of the protein to both oxytocin and vasopressin with similar contribution.

The nonapeptide hormones oxytocin and vasopressin are associated in the mammalian pituitary posterior lobes with a number of proteins, neurophysins.[3)] It has been assumed that neurophysins may be involved in the mechanisms for the storage and release of the hormones or they may act as carriers for the hormones in the hypothalamo-neurohypophysial

1) A part of this work was presented at the 95 th Annual Meeting of the Pharmaceutical Society of Japan, Nishinomiya, April 1975.

2) Location: *a) 1–18–1, Kamiyōga, Setagaya-ku, Tokyo, 158, Japan; b) 3–20–1, Kitashinjuku, Shinjuku-ku, Tokyo, 160, Japan.*

3) *a)* H.B. van Dyke, B.F. Chow, R.O. Greep, and A. Rothen, *J. Pharmacol. Exptl. Therap.*, **74**, 190 (1942); *b)* R. Acher, J. Chauvet, and G. Olivry, *Biochim. Biophys. Acta*, **22**, 421 (1956); *c)* R. Acher and C. Fromageot, "The Neurohypophysis," ed. by H. Heller, Academic Press Inc., New York, 1957, p. 39; *d)* C.R. Dean and D.B. Hope, *Biochem. J.*, **101**, 17p (1966); *e)* C.R. Dean and D.B. Hope, *ibid.*, **104**, 1082 (1967).

4) *a)* W. Bargmann, "The Neurohypophysis," ed. by H. Heller, Academic Press Inc., New York, 1957, pp. 11—22; *b)* W.H. Sawyer, *Pharmacol. Rev.*, **13**, 225 (1961).

system.[4] It has also suggested that neurophysins may be the remains of precursor from which the hormones are split off.[5] However, their true physiological role has been still obscure.

A hormone-protein complex can be extracted from pituitary posterior lobes. They are associated with noncovalent bonding and can be separated to each constituent by a number of mild procedures.[3b] Preparation of bovine neurophysin was further separated into two major and one minor constituents which have been termed neurophysin (NP) I, II, and C, respectively.[6,7] The unit structure of each of the bovine NPs has a molecular weight of approximately 10000 g and they resemble each other with respect to amino acid composition and to hormone-binding ability.[6] Examination of the partially known amino acid sequences of bovine NP-I and NP-II indicates obvious homologies except some substitutions.[8]

A number of binding studies using isolated NP and hormones or their peptide analogs have been done not only to elucidate the physiological role of hormone-NP complex but also because they provide useful and accessible models for the study of protein-protein interaction. Chemical modifications of hormones[9] or ultraviolet (UV),[10–12] circular dichroism (CD) difference,[11–15] and nuclear magnetic resonance (NMR)[16] spectroscopic studies have led to the conclusion that the hormone-protein interaction involved primary an electrostatic interaction between the protonated α-amino group of the hormones and unprotonated carboxyl group on the protein, which is enhanced by a nonpolar environment provided in part by the side chains in position 1—3 of the hormones and by unspecified residues on the protein. The direct involvement of the single tyrosine residue of the hormones in the binding process has also been well defined.

Although much information about the specific residues on the hormones, which directly participate in binding process, and the spectroscopic perturbation of some amino acid residues including tyrosine, phenylalanine, and cystine of protein on hormone binding have been available from above cited works, there are few direct evidence for the specific amino acid residues which constitute binding site of NP. Thus, it would be expected that selective modifications of amino acids at the binding site of the protein might allow more specific assignments. It has been already shown that nitration or O-acetylation of the tyrosine residue of NP-II could not affect the [8-arginine]vasopressin-binding ability of the modified protein.[10]

In the present work, purified bovine NP-II has been photooxidized in the presence of rose bengal and the relationships between the loss of hormones-binding ability of modified protein and the destruction of photosusceptible amino acids were examined.

5) H. Sachs and Y. Takabatake, *Endocrinology*, **75**, 943 (1964).
6) R. Rauch, M.D. Hollenberg, and D.B. Hope, *Biochem. J.*, **115**, 473 (1969).
7) M.D. Hollenberg and D.B. Hope, *Biochem. J.*, **106**, 557 (1968).
8) J.D. Capra, J.M. Kehoe, D. Kotelchuck, R. Walter, and E. Breslow, *Proc. Natl. Acad. Sci. U.S.*, **69**, 431 (1972).
9) *a*) J.E. Stouffer, D.B. Hope, and V. du Vigneaud, "Perspectives in Biology," ed. by C.F. Cori, V.G. Foglia, L.F. Leloir, and S. Ochoa, Elsevier Publishing Co., Amsterdam, 1963, p. 79; *b*) E. Breslow and L. Abrash, *Proc. Natl. Acad. Sci. U.S.*, **56**, 640 (1966); *c*) M. Camier, R. Alazard, and P. Cohen, *Eur. J. Biochem.*, **32**, 207 (1973).
10) A.J. Furth and D.B. Hope, *Biochem. J.*, **116**, 545 (1970).
11) E. Breslow and J. Weis, *Biochemistry*, **11**, 3474 (1972).
12) J.H. Griffin, R. Alazard, and P. Cohen, *J. Biol. Chem.*, **248**, 7975 (1973).
13) E. Breslow, H.L. Aanning, L. Abrash, and M. Schmir, *J. Biol. Chem.*, **246**, 5179 (1971).
14) E. Breslow, J. Weis, C.J. Menendez-Botet, and S. Lundt, *Federation Proc.*, **32**, 539 (1973).
15) E. Breslow, J. Weis, and C.J. Menendez-Botet, *Biochemistry*, **12**, 4644 (1973).
16) *a*) P. Balaram, A.A. Bothner-By, and E. Breslow, *J. Am. Chem. Soc.*, **94**, 4017 (1972); *b*) P. Balaram, A.A. Bothner-By, and E. Breslow, *Biochemistry*, **12**, 4695 (1973); *c*) P. Cohen, J.H. Griffin, M. Camier, M. Caizergues, P. Fromageot, and J.S. Cohen, *FEBS Lett.*, **25**, 282 (1972); *d*) J.A. Glasel, V.H. Hruby, J.F. McKelvy, and A.F. Spatola, *J. Mol. Biol.*, **79**, 555 (1973); *e*) R. Alazard, P. Cohen, J.S. Cohen, and J.H. Griffin, *J. Biol. Chem.*, **249**, 6895 (1974).

Experimental

Purification of NP-II——The method described by Hollenberg and Hope[7] for the preparation of bovine NPs was used with a few modifications. The acetone-dried powder of posterior lobes of bovine pituitary glands given by Teikoku Hormone MFG. Co., Ltd. was extracted with 0.1 N HCl at 4° for 24 hr. Insoluble material was removed by centrifugation at 0°, and was re-extracted with the solvent at 4° for 24 hr. Supernatants were combined and neutralized; the cloudy solution was centrifuged at 0°. The supernatant was decanted, adjusted to pH 3.9, and 15 g of NaCl were added to each 100 ml of the solution. Precipitation was allowed to proceed overnight in a cold-room at 4°, and the precipitate was collected by centrifugation at 0°. The sediment was taken up in water, dialysed against distilled water in order to remove salt, and then lyophilized. The lyophilized material was dissolved in 0.1 M pyridine acetic acid buffer, pH 5.7, and submitted to gel filtration on a column (5 × 75 cm) of Sephadex G-75 equilibrated with the pH 5.7 buffer. The NP-hormone complex fractions were collected, lyophilized, dissolved in 0.01 N HCl, adjusted to pH 2.0, and then submitted to gel filtration on a column (2.5 × 60 cm) of Sephadex G-25 equilibrated with 0.01 N HCl to remove hormones. The NPs fractions were collected, and lyophilized. Finally, the resulting substance was dissolved in 0.05 M Tris-HCl buffer, pH 8.1, and applied to a column (3 × 36 cm) of diethylaminoethyl (DEAE)-cellulose (DE-52, Whatman) equilibrated with 0.05 M Tris–HCl buffer, pH 8.1. The column was eluted at 20 ml/hr with the same buffer of increasing NaCl concentration linearly from 0 to 0.4 M. Protein was detected by measuring the absorbance of fractions at 280 nm. The fractions of main two peaks (tube No. 66—73 and 98—104) appeared in the chromatogram were found to be NP-II and NP-I fractions, respectively, by means of electrophoretical comparison of them with NP-II and I which have been prepared according to the procedure described by Hollenberg and Hope.[7] NP-II fractions were collected, concentrated with a rotary evaporator, dialysed against distilled water in order to remove salt, and then stored as a lyophilized powder.

Hormones——A solution of protein free hormones, oxytocin and [8-arginine]vasopressin, was prepared from the NP-hormone complexes by gel filtration on Sephadex G-25 at pH 2.0 as described above. The solution contained almost the same oxytocic and vasopressor activities (about 40 units per ml).

Determination of Hormones-Binding Ability——A solution (0.5 ml) containing 0.25 or 0.50 mg of protein was introduced in the inside of the 8/32 Visking tube and dialysed against 0.1 M phosphate buffer, pH 5.7, for 24 hr. This solution was then dialysed against a solution containing oxytocin and [8-arginine]vasopressin (each 1—2 units per ml) in 0.1 M phosphate buffer, pH 5.7, at 4° for more 48 hr with gentle shaking; the volume of fluid in the Visking tube was measured and the solutions inside and outside of the tube were assayed for oxytocic and vasopressor activities.

Bioassays——Oxytocin and vasopressin were assayed by the fowl blood pressure method and the spinal rat blood pressure method, respectively, detailed in the Pharmacopoeia of Japan, Eighth Edition.

Irradiation——Ten mg of NP-II were dissolved in 10 ml of 0.1 M phosphate buffer, pH 7.4, which contained 0.5 mg of rose bengal (National Institute of Hygienic Sciences Reference Standard) in the dark. The solution was then divided into two parts, one was kept in the dark as a control and the other put in a test tube stopped with a silicon rubber stopper to expose to the light. The tube was irradiated at 25 ± 3° by a 350 W reflecting photoflood lamp (Iwasaki Electric Co., Ltd.) at a distance of 15 cm from the reaction mixture, while the tube was gently shaken. Aliquots were removed from the irradiated solution at the desired time intervals.

Amino Acid Analysis——Usually, samples were hydrolysed without removing of rose bengal at 108° for 21 hr under vacuum in 6 N HCl. For the analysis of methionine, protein solutions desalted by dialysis against distilled water were lyophilized and then hydrolysed with 500 mg of $Ba(OH)_2 \cdot 8H_2O$ at 108° for 28 hr under vacuum. Barium was precipitated from the hydrolysate by a stream of CO_2. The analyses were performed on a Hitachi KLA-3B analyzer.

Acetylation——The acetylating agent was N-acetylimidazole (Seikagaku Kogyo Co., Ltd., Tokyo). The reagent was stored as a solution in benzene (200 μmoles/ml) dried with Na_2SO_4. The solid reagent (30 μmoles) obtained by evaporation of 0.15 ml of the reagent solution in a stream of dry air was dissolved in 1.25 ml of 0.01 M Tris–HCl buffer, pH 7.5, containing 0.5 μmoles of NP-II and the reaction was allowed to proceed for 1 hr at room temperature.

The O-acetylated protein was purified by chromatography on a column (1.3 × 25 cm) of Bio-Gel P-4 in 0.002 M Tris–HCl buffer (pH 7.5) and stored as a solution in concentration of 0.1 μmole of protein per ml. The procedures without adding of the acetylating reagent were also performed as a control.

De-O-acetylation——The acetylated protein or native protein solution (0.1 μmole/ml) was allowed to stand for 45 min at room temperature with an equal volume of 2 M hydroxylamine solution (pH 7.5). The de-acetylation was found to be finished completely in 45 min by means of absorption determination at 278 nm.

Results

Purification of NP-II

CM-Sephadex C-50 (Hollenberg and Hope[7]) and DEAE-Sephadex A-50 (Breslow and Abrash[9b]; Rauch, *et al.*[6]) have been used as the final step of purification procedure of bovine

NPs. In the present work DEAE-cellulose (DE-52, Whatman) was used for the purification of NP-II. Figure 1 shows a typical chromatogram on DEAE-cellulose of hormone free NPs obtained by gel filtrations of crude NP-hormone complexes on Sephadex G-75 and G-25. NP-I and NP-II were found in the fractions of main two peaks, (b) (tube No. 98—104) and (a) (tube No. 66—73), in Fig. 1, respectively, which were confirmed by disc electrophoresis with authentic samples. Although NP-I obtained directly from peak(b) was still not homogeneous, NP-II from peak(a) was electrophoretically homogeneous. Electrophoretically homogeneous NP-I was obtained after the peak(b) fraction was subjected to re-chromatography on the same column (Fig. 2).

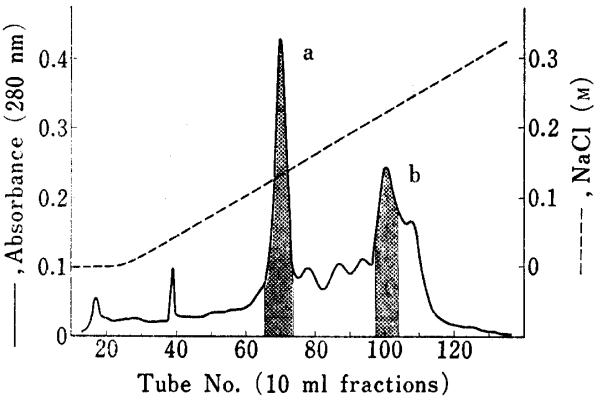

Fig. 1. Ion Exchange Chromatography of Bovine Neurophysins on DEAE-Cellulose

The crude hormone free neurophysins fraction (600 mg) obtained by gel filtrations through Sephadex G-75 and G-25 of an extract of pituitary posterior lobes was applied to a column (3 × 36 cm) of DE-52 (Whatman) equilibrated with 0.05M Tris-HCl buffer (pH 8.1) and the column was eluted at 20 ml/hr with the above buffer of increasing NaCl concentration gradiently from 0 to 0.4M. Neurophysin I and II were found in the fraction (b) (tube No. 98—104) and (a) (tube No. 66—73), respectively.

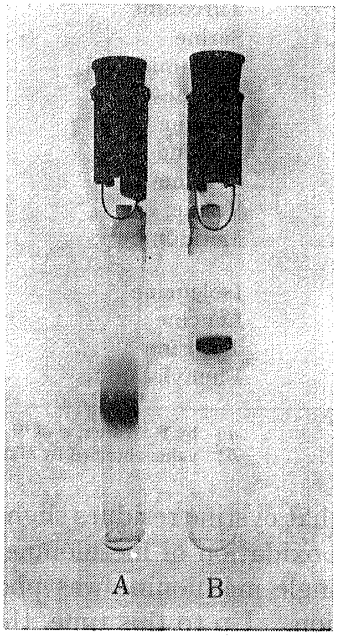

Fig. 2. Disc Electrophoretograms of Neurophysin I (A) and II (B) Fractions obtained from Fig. 1

Electrophoresis was performed in 7.0% polyacrylamide gel as described by Ornstein and Davis.[17] Proteins were stained with 0.5% Amido Black 10B in 7.5% (v/v) acetic acid.

Photooxidation of Amino Acid Residues of NP-II in the Presence of Rose Bengal

Table I shows the changes in amino acid compositions as a result of photooxidation of bovine NP-II in the presence of rose bengal. After 120 min of irradiation, single methionine at position 2 and single tyrosine at position 49[18] were found to be photooxidized completely. The photoproduct of methionine may be characterized to be methionine sulphoxide since no appreciable amounts of methionine were found in alkaline hydrolysate of photooxidized NP-II while approximately 1 mole of methionine was recovered after acid hydrolysis.[19] Jori, *et al.* have shown that methionine is converted to the sulphoxide on illumination in air with rose bengal as a sensitizer.[20] On the other hand, the photooxidation of tyrosine is thought to involve rupture of the ring.[21] There occur no significant changes in other amino acids

17) L. Ornstein, *Ann. N. Y. Acad. Sci.*, **121**, 321 (1964); B.J. Davis, *ibid.*, **121**, 404 (1964).
18) R. Walter, D.H. Schlesinger, I.L. Schwartz, and J.D. Capra, *Biochem. Biophys. Res. Commun.*, **44**, 293 (1971).
19) W.J. Ray, Jr. and D.E. Koshland, Jr., *J. Biol. Chem.*, **237**, 2493 (1962).
20) G. Jori, G. Galiazzo, A. Marzotto, and E. Scoffone, *Biochim. Biophys. Acta*, **154**, 1 (1968).
21) L. Weil, W.G. Gordon, and A.R. Buchert, *Arch. Biochem. Biophys.*, **33**, 90 (1951); S. Gurnani and M. Arifuddin, *Photochem. Photobiol.*, **5**, 341 (1966).

Table I. Amino Acid Composition of Neurophysin II photooxidized in the Presence of Rose Bengal

Amino acid	Reported[a]	Found Irradiated for 0 min	Found Irradiated for 120 min
Lysine	2	2.1	2.2
Arginine	7	7.0	7.0
Aspartic acid	5	5.1	4.8
Threonine	2	2.0	1.9
Serine	6	5.7	5.7
Glutamic acid	14	14.3	14.2
Proline	8	8.0	7.9
Glycine	16	15.8	16.0
Alanine	6	6.4	6.4
Cystine	7	6.7	6.3
Valine	4	3.4	3.4
Methionine	1	0.92	0.86 (0.0)[b]
Isoleucine	2	2.2	2.3
Leucine	6	6.2	6.1
Tyrosine	1	0.91	0.0
Phenylalanine	3	3.0	3.0

a) by R. Walter, *et al* [18]
b) value obtained by alkaline hydrolysis

except that cystine residues slightly tend to be photooxidized. Figure 3 shows the time course of photooxidation of methionine, tyrosine, and cystine residues of NP-II. Under the conditions, single methionine was photooxidized very rapidly and it disappeared within 7 min of irradiation. Up to this time, the photodestruction of single tyrosine residue was found to be only about 20% and 120 min of irradiation were required for its complete destruction. The photooxidation of cystine residues proceed very slowly; 120 min of irradiation result in only about 6% decomposition and even when prolonged irradiation (240 min) was carried out only about 10% of original cystine residues were photooxidized. Such remarkable differences in photosusceptibility of various amino acid residues in NP-II seem to allow the selective modification of specific amino acid residues by setting the irradiation time suitably. It would be expected, therefore, that this photochemical technique may serve as useful means for the elucidation of the functional role of some amino acid residues in NP-II.

Hormones-Binding Ability of Photooxidized NP-II

To determine the effect of photochemical modification of methionine, tyrosine, and cystine residues in NP-II on the biological activity of the protein, the hormones-binding ability was tested by equilibrium dialysis. Table II shows the changes in binding ability of NP-II for oxytocin and [8-arginine]vasopressin as a result of photooxidation in the presence of rose bengal. Evidently, the hormones-binding ability of the protein was decreased with time of irradiation. It is of interest that a decrease in binding ability of the modified protein proceeds almost identically for oxytocin and vasopressin as the ligand. The relationships between the degree of inactivation of NP-II and the degree of photochemical modification of methionine, tyrosine, and cystine residues are shown in Fig. 4. It is clearly indicated that single methionine residue of NP-II is nonessential in the binding process since the hormones-binding ability of the protein is almost completely retained even when the residue was completely photooxidized. It is also evident that the photochemical modification of single tyrosine residue has some relevance to a decrease in hormones-binding ability of the protein. However, a progressive decrease in binding ability with increasing photodegradation of tyrosine residue was observed

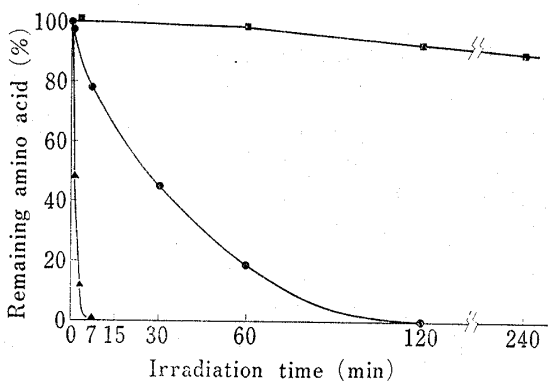

Fig. 3. Photooxidation Rate of the Methionyl (▲), the Tyrosyl (●), and the Cystyl (■) Residues of Bovine Neurophysin II on Irradiation at 25±3° in 0.1M Phosphate Buffer (pH 7.4) in the Presence of Rose Bengal

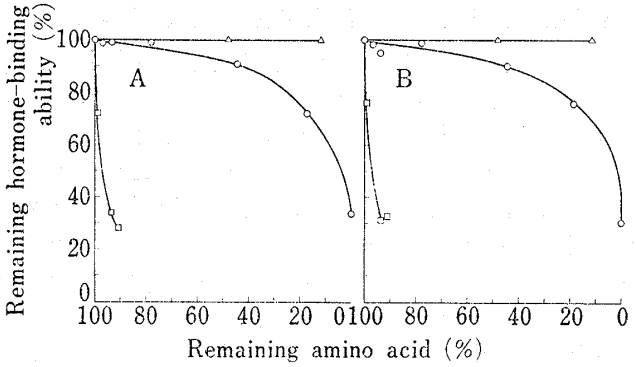

Fig. 4. Relationship between Remaining Per Cent of the Methionyl (△), the Tyrosyl (○), or the Cystyl (□) Residue and That of Oxytocin(A)- or Vasopressin(B)-Binding Ability of Bovine Neurophysin II upon Irradiation in 0.1M Phosphate Buffer (pH 7.4) in the Presence of Rose Bengal

TABLE II. Hormones-Binding Abilities of Neurophysin II Photooxidized in the Presence of Rose Bengal

Irradiation time (min)	Oxytocin		Vasopressin	
	u/mg of protein	(%)	u/mg of protein	(%)
0	10.02	100	10.54	100.0
7	9.94	99.2	10.54	100.0
15	9.56	95.4	9.60	91.1
30	9.04	90.2	9.50	90.1
60	7.08	70.7	8.02	76.1
120	3.34	33.3	3.24	30.8
240	2.66	26.6	3.48	33.0

Bound hormones activities were estimated by the bioassay after the proteins were dialyzed against a solution of oxytocin and [8-arginine] vasopressin in 0.1M phosphate buffer, pH 5.7.

rather than a linear relationship. For the explanation of this phenomenon the influence of the photooxidation of cystine residues on the hormones-binding ability of the protein may have to be taken into account. However, the findings that about 30% of the original hormones-binding ability had been lost at the time when the photooxidation of cystine residues scarcely occurred (Fig. 4) and that prolonged irradiation of 2 hr after the complete destruction of tyrosine residues, in which the photooxidation of cystine residues proceeded from 6 to 10% (Fig. 3), results in no significant loss of hormones-binding ability of the protein (Table II) may exclude the possibility of the direct involvement of cystine residues in binding process. It may generally be considered that cystine residues serve as the backborn to maintain the secondary or tertiary structure of the protein or/and to allow the reversible conformational changes favorable to biological function of the macromolecular. Thus, it is most probable that the photooxidation of cystine residues may result in the partial destruction of secondary or tertiary structure of the protein or/and the reduction of the flexibility of the protein to the conformational changes. Such situation may provide some effect, in addition to the photo-destruction of tyrosine residue, on the loss of hormones-binding ability of the protein. Since the further photooxidation of cystine residues after the complete destruction of single tyrosine residue did not affect the hormones-binding ability of the protein (Table II), it seems likely that the influence of the photooxidation of cystine residues on the binding ability of the protein may exist in the enhancement of the influence resulting from the photodestruction of tyrosine residue. This suggests the close correlationships between the function of tyrosine-49 and the flexible conformational changes of the protein in the binding process.

pH Binding Profiles of Photooxidized NP-II

In attempts to elucidate the role of single tyrosine of NP-II, the binding of both oxytocin and [8-arginine]vasopressin to the photooxidized NP-II in which single tyrosine has been lost was investigated over the pH range 4.0—7.5. As shown in Fig. 5, the pH binding profiles of photooxidized NP-II for both hormones are found to be essentially identical with those of native protein. Since the pH binding profiles of native NP-II have been thought to reflect the electrostatic interaction between hormones and NP-II, it seems likely that single tyrosine residue does not contribute to the electrostatic component of the interaction. This is in accordance with the findings that acetylation of phenolic hydroxyl group of single tyrosine residue of NP-II can not affect the hormones-binding ability of the protein (Table III, see also reference (10)).

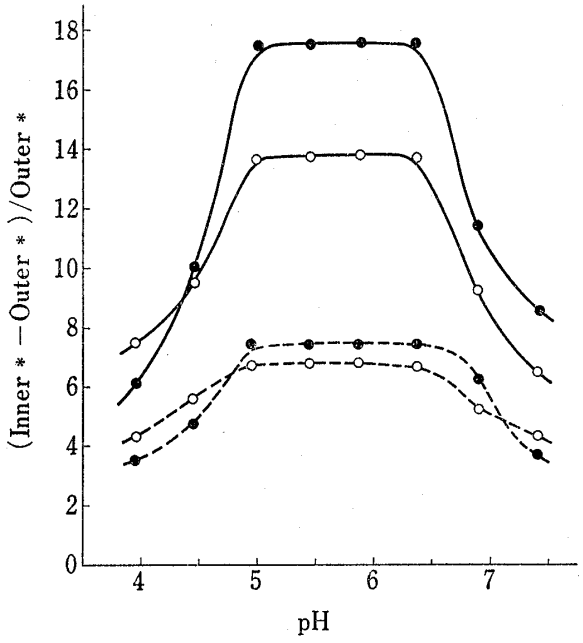

Fig. 5. Binding of Oxytocin and Vasopressin to Native and Photooxidized Neurophysin II as a Function of pH

——: native neurophysin II
----: oxidized neurophysin II
○: oxytocin, ●: vasopressin
*: hormone activity (u/ml) of inner or outer solution in the equilibrium dialysis

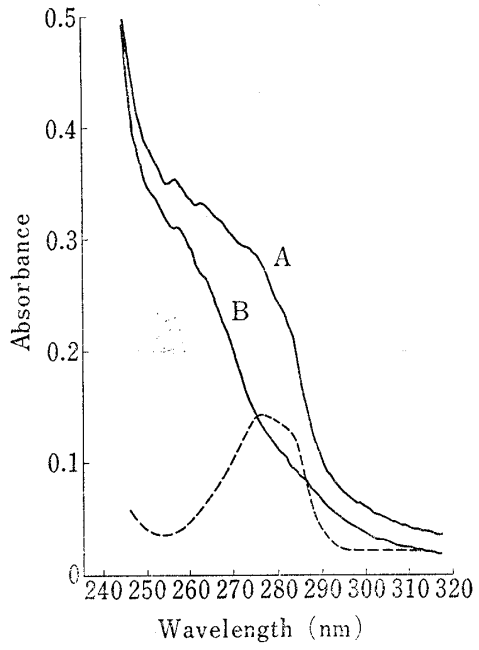

Fig. 6. Spectra of Native (curve A) and O-Acetylated (curve B) Neurophysin II at pH 7.5

The final concentration of protein (0.1 μmole) was identical for the two spectra. Dotted line is the difference spectrum due to the acetylation.

Acetylation of NP-II

It has revealed that the photooxidation of single tyrosine residue of NP-II led to the significant loss of the hormones-binding ability of the protein. On the other hand, it has been shown that nitration or O-acetylation of the tyrosine residue of NP-II could not affect the [8-arginine]vasopressin-binding ability of modified protein.[10] In the present study an examination was made whether or not O-acetylation of tyrosine residue of NP-II affect the binding ability of the protein for both oxytocin and [8-arginine]vasopressin. Acetylation of NP-II was carried out using a 60-fold molar excess of N-acetylimidazole at pH 7.5 and room temperature (about 20°) for 1 hr. The extent of O-acetylation of the protein was followed spectrophotometrically after the removal of excess reagent by gel filtration with Bio-Gel P-4 column. Acetylation of NP-II produces a characteristic decrease in absorption between 250 and 300 nm (Fig. 6), as expected to O-acetylation of the tyrosine residue.[10,22] The dotted

22) J.F. Riordan, W.E.C. Wacker, and B.L. Vallee, *Biochemistry*, 4, 1758 (1965).

line in Fig. 6 shows the difference spectrum due to acetylation of NP-II, indicating a tyrosine like spectrum. When the decrease in absorbance at 278 nm, $\Delta\varepsilon_{278}=1160$ per mole, on O-acetylation of N-acetyltyrosine[22] has been used to determine the number of tyrosyl residues modified in protein, 1.1 moles of O-acetyltyrosine was found per 10000 g of protein. Upon treatment of the acetylated NP-II with 2M hydroxylamine at pH 7.5, the restoration of ultraviolet (UV) absorption was observed. Tyrosyl deacetylation was determined from the increase in absorbance at 278 nm using a factor of 1160 per tyrosyl residue per mole and was found to be 1.1 moles per mole of protein. From these results it is evident that single tyrosine residue of NP-II was acetylated almost completely under the conditions.

Table III. Effect of Tyrosine O-Acetylation on the Hormones-Binding Ability of Bovine Neurophysin II

Neurophysin II	Oxytocin units bound to 0.25 mg of protein	Average	%
Native	1.65;1.74	1.70	100
Acetylated	1.83;1.80	1.82	107.1
Native+Hydroxylamine	1.25;1.23	1.24	100
Acetylated+Hydroxylamine	1.40;1.34	1.37	110.5

Neurophysin II	Vasopressin units bound to 0.25 mg of protein	Average	%
Native	1.23;1.15	1.19	100
Acetylated	1.30;1.33	1.32	110.9
Native+Hydroxylamine	1.07;1.11	1.09	100
Acetylated+Hydroxylamine	1.26;1.19	1.22	111.9

Experimental details of acetylation and O-deacetylation of neurophysin II are as described in the "Experimental". Hormones-binding ability was tested by equilibrium dialysis against a solution of oxytocin and [8-arginine] vasopressin in 0.1M phosphate buffer (pH 5.7). In each experiment the hormones-binding ability of the native protein was tested under identical conditions and with the same solution of hormones as for the modified protein.

Table III shows the binding ability of acetyl NP-II and O-deacetylated preparation for both oxytocin and [8-arginine]vasopressin. Although acetylation of NP-II led to a slight increase in hormones-binding ability, there occur no significant changes in binding ability as a result of O-deacetylation of tyrosine residue of the acetylprotein. This, together with the results of the binding study of acetyl NP-II,[10] CD and NMR spectroscopic studies,[11,16b] and pH binding profiles of photooxidized NP-II, indicates that the phenolic hydroxyl group of tyrosine-49 of NP-II may not involve in the binding interaction between NP-II and peptide hormones.

Discussion

Upon irradiation of bovine NP-II with visible light in the presence of rose bengal and oxygen, single methionine at position 2 of the protein was rapidly photooxidized, giving mainly methionine sulphoxide (Table I, Fig. 3). The photooxidation of methionine-2, however, could not affect the binding ability of the protein for both oxytocin and vasopressin (Fig. 4). Breslow, *et al.* has already pointed out that the lone methionine of bovine NP-II is unessential to its binding activity.[13] Their conclusion has been drawn from the findings that amino acid composition of one of the principal protein fractions isolated by dilute H_2SO_4 extraction of acetone–dried posterior pituitary powder is identical with NP-II except for the absence of one alanine and the single methionine[13] and that this protein fraction has normal ability to bind oxytocin and vasopressin.[9b] The present studies on the photooxidation of methionine-2 of NP-II may also provided a strong supporting evidence for the nonessential role of single methionine of NP-II for hormones-binding.

It is of particular interest for the role of single tyrosine residue of NP-II on the hormones-binding process. Furth and Hope have shown that neither nitration nor O-acetylation of the tyrosine residue of NP-II led to any fall in the binding capacity of the protein for [8-arginine]-vasopressin.[10] CD and NMR studies of the interaction of hormones and their peptide analogs with NP-II or nitrated NP-II also suggest a nonessential role for the protein tyrosine hydroxyl in the binding interaction and that nitration does not block the binding.[11,16b] In the present work it is also confirmed that O-acetylation of single tyrosine residue of NP-II could not affect the binding ability of NP-II for both oxytocin and vasopressin. However, photooxidative destruction of tyrosine residue by the action of rose bengal led to the significant loss in binding ability of NP-II for both hormones. This is a first experimental evidence that a modification of single tyrosine of NP-II affect the hormones-binding ability of the protein. That the different methods for modification of tyrosine residue of NP-II produce the dissimilar effect on the biological activity of the protein may be attributable to the differences in the product resulting from the individual modification method. The aromatic ring has been believed to be cleaved in the dye-sensitized photooxidation of tyrosine,[21] while the aromatic ring has been still retained either after O-acetylation or mononitration of tyrosine. Therefore, the findings that the significant loss of hormones-binding ability is first caused by the photooxidative destruction of tyrosine residue of NP-II suggest that the tyrosine residue may contribute to hormones-binding ability of the protein through its aromatic ring.

There are some studies which demonstrate that the microenvironment of tyrosine-49 of NP-II is changed by hormone binding. Interaction of nitrated NP-II and [8-arginine]-vasopressin gave rise to a characteristic difference spectrum with a peak at 436 nm,[10] and this shows that the tyrosine of the protein is perturbed. From the very large shift (84 nm) in this region Furth, *et al.* suggested that 3-nitrotyrosyl residue not only enters a more hydrophobic environment on protein–hormone interaction, but is caused to ionize more fully.[10] Spectrophotometric titration studies of nitrotyrosine in nitrated NP-II in the presence or absence of oxytocin, [8-arginine]vasopressin, or their peptide analogs indicated that the pK of the nitrotyrosine of the hormone complex, 6.9, was lower than that of the uncomplexed nitrated protein, 7.45, which is abnormally high.[11] CD studies of nitrated NP-II also showed an increased ionization of the nitrotyrosine in the complex,[11] whereas nitrotyrosine ellipticity changes attendant to hormone binding seemed to exclude the previous assumption that nitrotyrosyl residue of protein enters a more hydrophobic environment on protein-hormone interaction.[10] The high pK of the nitrotyrosine in noncomplexed protein was explained as the proximity of a negative group which can only be a carboxyl.[11] The diminution in pK of the nitrotyrosine on hormone binding was also interpreted to be attributable to the proximity of the protonated α-amino group of the bound peptide whose influence in part is to neutralize the negative charge on a nearby carboxyl.[11] NMR studies have shown that on binding with hormone tyrosine-49 in NP-II becomes more deshielded and experiences a greater freedom of motion (or is subject to fewer dipolar interactions).[16a,b] NMR results also suggest, together with the data obtained from the spectral and CD studies,[10,11] that either the protein tyrosine is displaced from an intramolecular binding interaction by the entering peptide, or that it is freed from motional constraints by a local conformational change attendant to binding. In the limitation of such observations, however, it has been uncertain whether or not tyrosine-49 in NP-II participates in binding process directly. Thus, the present findings that the intact aromatic ring of tyrosine-49 in NP-II may participate in the hormones binding process seem to be brought new insight in the studies of hormones-NP-II interactions.

Since aromaticity and hydrophobicity in position 2 of both the hormones and their peptide analogs were apparently required for their binding to NP-II,[15] some relevance of the aromaticity of protein tyrosine and that of hormone tyrosine may be implied. In fact, CD[11] and NMR data[16a,b] suggest that protein tyrosine residue is spatially proximate to the aromatic ring at position 2 of the bound peptide. However, the absence of stacking interactions[16a,b]

or of π–π interactions[16e] between those tyrosine rings in the complex can be inferred from the NMR studies. Alternatively, for the explanation of the apparent requirements for aromaticity and hydrophobicity in position 2 of hormones and aromatic ring in position 49 of NP-II following situation may tentatively be taken into consideration: tyrosine in position 2 of hormones can bind a site of NP-II, which possess a steric requirement for a hydrophobic residue which is planar[15]; in uncomplexed NP-II, tyrosine-49 is situated in this site with an intramolecular interaction with a proximal carboxyl group which would be expected to form an electrostatic bond with the protonated α-amino group of the hormones on binding; in the presence of hormones, the protein tyrosine is, simultaneously, displaced from such planar hydrophobic region by the entering tyrosine of the hormones and freed from the intramolecular binding interaction by the neutralization of a proximal carboxyl group on the protein through the formation of ion pair with protonated α-amino group of the hormones. A number of observations that upon the complex formation the microenvironment of hormones tyrosine and that of protein tyrosine change each other in a contrary manner such that hormones tyrosine moves from less to more shielded and hydrophobic environments[9b,10,11,13,15,16] while protein tyrosine moves from less to more deshielded and polar environments[10,11,16a,b,e] may have used as an argument for the displacement of protein tyrosine and hormone tyrosine in binding site of NP-II.

In connection with this it should be noted that positive cooperativity in binding exists between residue 1 (α-amino group) and residue 2 (tyrosine) of hormones.[15] It seems likely that similar cooperation of tyrosine-49 and the carboxyl group which serve as a binding constituent may exist and provide the principal driving force for hormone binding. It is possible that, in protein, the movement of tyrosine residue as a result of the breaking of the intramolecular interaction with the carboxyl group, which seemed to be induced, as cited above, by approaching the hormone peptide, mainly its α-amino group and probably also tyrosine-2, constrains the protein into more favorable conformation for binding and it may also provide a sterically acceptable binding site for hormones tyrosine which is planar. If so, the destruction of tyrosine in protein results in the loss of the cooperative factor of the tyrosine and nearby carboxyl group which existed in native protein. This would be expected to lead to weaking of binding ability of NP-II.

The findings that the influence of the destruction of tyrosine-49 of NP-II on the loss of binding ability of the protein was amplified by the photooxidation of cystine residues may also suggest the correlativity of a function of tyrosine-49 and suitably flexible protein conformation for which cystine residues certainly serve as backborn. A change in disulfide environment or geometry of NP-II on binding has been deduced from CD studies[11] though the direct involvement of cystine residues of NP-II in binding process is unlikely because that direct correlation between photooxidation of cystine residues and the loss of binding ability of NP-II could not be found in the present experiment (Fig. 4).

It is noteworthy that the photooxidation of NP-II led to almost identical loss of its binding ability for oxytocin and vasopressin. Since competitive experiments between oxytocin and vasopressin showed that each compound inhibits the binding of the other peptide in a non-competitive fashion, partial overlap of binding site of oxytocin and that of vasopressin has been postulated.[9c] It is, therefore, highly probable that tyrosine-49 of NP-II may participate with binding process of both oxytocin and vasopressin to protein, commonly, with similar contribution. This is consistent with the findings that the single tyrosine of NP-II are almost identically perturbed on addition of single equivalents of oxytocin and vasopressin.[15]

Further studies on the hormones binding sites of NP-II and the role of the single tyrosine of the protein are in progress and will be reported elsewhere.

Acknowledgement The authers wish to thank Teikoku Hormone MFG. Co., Ltd. for generous supplies of bovine posterior pituitary glands.