The Chemical Modifications and Their Effects on the Hormone-Binding Ability of Bovine Neurophysin I[1]

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In order to elucidate the functional role of single amino acid residue, histidine or tyrosine of neurophysin I for the binding ability of the protein, these amino acids have been modified photochemically or chemically and their effects on the binding abilities for oxytocin and [8-arginine]vasopressin have been investigated.

Upon irradiation of NP-I with visible light in the presence of rose bengal and oxygen, single histidine residue was photooxidized very rapidly without any decrease in the hormones-binding ability. On the other hand, single tyrosine residue was found to be photooxidized almost completely after 240 min of irradiation with decrease in the hormone-binding ability. Neither significant changes in other amino acid residues nor peptide bond rupture were found even after 240 min of irradiation.

The decrease in the binding ability of the photooxidized protein proceeds almost identically for oxytocin and vasopressin as the ligand.

O-Acetylation of the tyrosine residue of NP-I with N-acetylimidazole gives no significant effects on the hormone-binding ability.

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

In the mammalian pituitary posterior lobes two kinds of peptide hormones, oxytocin and vasopressin, are associated with a number of proteins, neurophysins (NP). Bovine NP were found to be separated into two major and one minor constituents which have been termed neurophysin I, II and C, respectively.[3] Although several works on the physiological role of NP have been done,[4,5] the significance of the existence of several hormone-binding proteins has been still obscure. Studies on the interaction between each isolated neurophysin and the hormones may contribute to our understanding of the physiological role of NP and the structure of the hormone as it exists in the neurosecretory granules. This may also provide a model for binding of peptide hormones to proteins.

A number of studies using purified NP-I or NP-II have led to the conclusion that the hormone-protein interaction involves primarily an electrostatic interaction between the protonated α-amino group of the hormones and an unprotonated side-chain carboxyl group

1) A preliminary account of some of this work has appeared in Chem. Pharm. Bull. (Tokyo), 23, 2184 Tokyo, 192-03, (1975).
2) Location: a) 1-18-1, Kamiyōga, Setagaya-ku, Tokyo, 158, Japan; b) 1432-1, Horinouchi, Hachiōji, Tokyo, 192-03, Japan.
3) M.D. Hollenberg and D.B. Hope, Biochem. J., 106, 557 (1968); C.R. Dean and D.B. Hope, ibid., 106, 565 (1968); R. Rauch, M.D. Hollenberg, and D.B. Hope, ibid., 115, 473 (1969).
4) W. Bargmann, "The Neurohypophysis," ed. by H. Heller, Academic Press Inc., New York, 1957, pp. 11—22; W.H. Sawyer, Pharmac. Rev., 13, 225 (1961).
5) C.R. Dean, D.B. Hope, and T. Kažić, Brit. J. Pharmacol., 34, 192p (1968); E.A. Zimmerman, A.G. Robinson, M. Kazim Husain, M. Acosta, A.G. Frantz, and W.H. Sawyer, Endocrinology, 95, 931 (1974).

of the protein in the midst of a hydrophobic environment.[6] Although there are some evidences of direct participation of some other sites on the hormones in the binding process[6] there are few direct evidence for the specific amino acid residues, which constitute binding site of NP. An interesting feature of isolated bovine NP-I and II is that they differ in amino acid composition but display similar hormone binding properties.[7] Examination of the partially known amino acid sequences of NP-I and II indicates obvious homologies except some substitutions.[8] Thus it is of particular interest to examine the contribution of these common structures as well as dissimilar parts to hormone-binding ability of the two different proteins. NP-I and NP-II both have single tyrosine residue at position 49 in each molecular and the former possesses single histidine and no methionine residue, while the latter possesses single methionine at position 2 but no histidine residue.[6g] The informations on the role of these single amino acid residues in these proteins for the hormones-binding ability would help us to consider the binding site of NP. Our previous findings have shown the possibility of selective photochemical modification of the methionine and tyrosine residues of bovine NP-II by the action of rose bengal as photosensitizer and have also indicated the unessential role of methionine-2 and the possible role of tyrosine-49 in the hormone binding process.[9]

In the present work, such photochemical technique is applied for the selective modification of histidine and tyrosine residues of NP-I and its effects on hormones-binding ability of the protein are examined. The influences of the O-acetylation of tyrosine-49 on the binding ability are also studied.

Experimental

NP-I——The method described previously for the preparation of bovine NP was used to obtain NP-I.[9] The acetone dried powder of posterior lobes of bovine pituitary glands given by Teikoku Hormone MFG. Co., Ltd., Tokyo was extracted with 0.1 N HCl. NP-hormone complexes were precipitated with 15% NaCl from the extract and then purified by gel filtration on Sephadex G-75 after dissolved the sediment in 0.1 M pyridine-acetic acid buffer, pH 5.7. The NP-hormone complexes fraction was submitted to gel filtration on Sephadex G-25 at pH 2.0 in order to separate hormones from NP. The hormone free NP fraction was then submitted to chromatography on DEAE-cellulose (ED-52, Whatman) and NP-I fraction (corresponding to peak b, see Fig. 1 in reference 9) was collected. Electrophoretically homogeneous NP-I was gained after rechromatography of the NP-I fraction on DEAE-cellulose in the same conditions.

Hormones——A solution of NP free hormones, oxytocin and [8-arginine]vasopressin, was prepared 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 0.5 mg of protein was introduced in the inside of 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 0.1M phosphate buffer, pH 5.7, at 4° for more 48 hr with gentle shaking; the volume of fluid in

6) 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); d) A.J. Furth and D.B. Hope, *Biochem. J.*, 116, 545 (1970); e) E. Breslow and J. Weis, *Biochemistry*, 11, 3474 (1972); f) J.H. Griffin, R. Alazard, and P. Cohen, *J. Biol. Chem.*, 248, 7975 (1973); g) E. Breslow, H.L. Aanning, L. Abrash, and M. Schmir, *J. Biol. Chem.*, 246, 5179 (1971); h) E. Breslow, J. Weis, C.J. Menendez-Botet, and S. Lundt, *Fed. Proc.*, 32, 539 (1973); i) E. Breslow, J. Weis, and C.J. Menendez-Botet, *Biochemistry*, 12, 4644 (1973); j) P. Balaram, A.A. Bothner-By, and E. Breslow, *J. Am. Chem. Soc.*, 94, 4017 (1972); k) P. Balaram, A.A. Bothner-By, and E. Breslow, *Biochemistry*, 12, 4695 (1973); l) P. Cohen, J.H. Griffin, M. Camier, M. Caizergues, P. Fromageot, and J.S. Cohen, *FEBS Lett.*, 25, 282 (1972); m) J.A. Glasel, V.H. Hruby, J.F. McKelvy, and A.F. Spatola, *J. Mol. Biol.*, 79, 555 (1973); n) R. Alazard, P. Cohen, J.S. Cohen, and J.H. Griffin, *J. Biol. Chem.*, 249, 6895 (1974): o) J.H. Griffin, J.S. Cohen, P. Cohen, and M. Camier, *J. Pharm. Sci.*, 64, 507 (1975).
7) E. Breslow and R. Walter, *Mol. Pharmacol.*, 8, 75 (1972).
8) J.D. Capra, J.M. Kehoe, D. Kotelchuck, R. Walter, and E. Breslow, *Proc. Natl. Acad. Sci. U. S.*, 69, 431 (1972).
9) H. Fukuda, T. Hayakawa, J. Kawamura, and Y. Aizawa, *Chem. Pharm. Bull.* (Tokyo), 24, 36 (1976).

the Visking tube was measured and the solutions both inside and outside of the tube were assayed for oxytocic and vasopressor activities by bioassays.

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-I 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 $25 \pm 3°$ 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 constant boiling HCl (6 N). The analyses were performed on a Hitachi KLA-3B amino acid analyzer.

Electrophoresis——Disc-electrophoresis on 15% polyacrylamide gels with 0.1% sodium dodecyl sulfate in the presence of 5% β-mercaptoethanol was performed according to the method of Laemmli.[10]

O-Acetylation and De-O-acetylation——Selective acetylation of tyrosine residue of NP-I was carried out in conjunction with the reversible blocking of amino groups by citraconylation.

The acetylating agent, N-acetylimidazole (Seikagaku Kogyo Co., Ltd., Tokyo), was stored as a solution in benzene (200 μmoles/ml) dried with Na_2SO_4. A sample of NP-I (10.8 mg) was dissolved in 10 ml of 0.1 M sodium borate buffer, pH 8.0, and 50 μl of citraconic anhydride (Seikagaku Kogyo Co., Ltd., Tokyo) was added over a 20 min period. The pH was maintained at 8.0 by addition of 4 M NaOH. Citraconylation was carried out for a total of 40 min. The pH was lowered to 7.5 with 1 N HCl and the solution was added to the solid N-acetylimidazole (50 μmoles) obtained by evaporation of the reagent solution in a stream of dry air. After 1 hr the pH was lowered to 3.5 and the mixture was stirred for 17 hr to remove the reversible blocking groups. For the removal of excess reagents the solution containing O-acetylated protein was concentrated to about 2 ml by ultrafiltration through membrane filter (Diafilter, G-05T, Bioengineering Co., Ltd., Tokyo) and submitted to gel filtration on a column (1.3×30 cm) of Bio-Gel P-4 (100—200 mesh) equilibrated with 0.1 M formic acid. The O-acetylated protein was stored as a freeze-dried powder (6.7 mg).

De-O-acetylation was accomplished by treatment of modified protein solution with 1 M hydroxylamine at pH 7.5. A solution of hydroxylamine (2 M) at pH 7.5 was prepared by dissolving 2.78 g of hydroxylamine (Kishida Kagaku Co., Ltd., Osaka) in 20 ml of 2 M NaOH. A sample of acetylated protein (3.0 mg) was dissolved in 1.5 ml of 0.1 M phosphate buffer, pH 7.5, and to the solution were added 1.5 ml of 2 M hydroxylamine and left for 45 min at room temperature. The mixture was dialyzed against distilled water in order to remove reagents prior to determination of the hormone-binding ability of de-O-acetylated protein.

Ultraviolet Spectra——Ultraviolet spectra of O-acetylated and de-O-acetylated protein were measured with a Hitachi 356 two-wavelength double beam spectrophotometer with 1 cm silica cells in the protein concentration of 0.1 μmole/ml at pH 7.5.

Results

Photochemical Modification of Amino Acid Residues of NP-I in the Presence of Rose Bengal

NP-I contains single histidine and single tyrosine, both which are photosusceptible amino acids, in its molecule. It has been generally accepted that histidine residues are destroyed fastest in most proteins by photodynamic treatment and that they oxidized more selectively when rose bengal was used as a sensitizer.[11] The destruction of tyrosine residues very often does not occur, or if it does occur, does not start until most of the available histidine residues are oxidized.[12] Thus the photochemical modification using rose bengal was used as a first choise for the studies on the structure and function of these single amino acid in NP-I. Fig. 1 shows the time course of photooxidation of histidine and tyrosine residues of NP-I. Under the conditions, single histidine was photooxidized very rapidly and it disappeared within 3 min of irradiation. At this time, no significant photodestruction of single tyrosine was observed and 120—240 min of irradiation were required for its complete destruction. There occurred no significant changes in other amino acid residues including cystine residues even after 240 min of irradiation (Table I). Such remarkable difference in photosusceptibility

10) U.K. Laemmli, *Nature*, **227**, 680 (1970).

11) E.W. Westhead, *Biochemistry*, **4**, 2139 (1965).

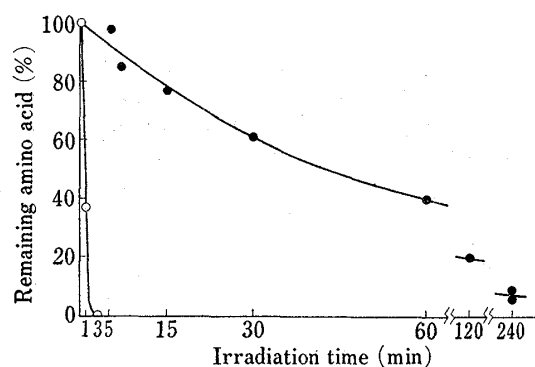12) J.D. Spikes and R. Livingston, *Advan. Radiation Biol.*, **3**, 29 (1969).

Fig. 1. Photooxidation Rate of the Histidyl
(○) and the Tyrosyl (●) Residues of
Bovine Neurophysin I on Irradiation at
$25 \pm 3°$ in 0.1M Phosphate Buffer (pH 7.4)
in the Presence of Rose Bengal

of histidine and tyrosine residues in NP-I under
the experimental conditions would be suitable
for the elucidation of the functional role of
these amino acid residues in NP-I.

It is generally considered that all photo-
dynamic damage to proteins results from the
destruction of amino acid side chains and, in
fact, there is no evidence for the rupture of
peptide bonds.[12] To test for the possibility
of the rupture of peptide bond in NP-I during
the irradiation, NP-I irradiated for 240 min was-
submitted to SDS-gel electrophoresis. Poly-
acrylamide gel electrophoresis in 0.1% sodi-
um dodecyl sulfate in the presence of 5%
β-mercaptoethanol showed that the photooxi-
dized protein migrated as a substance having

TABLE I.   Amino Acid Composition of Neurophysin I
Photooxidized in the Presence of Rose Bengal

Mole numbers were calculated based on arginine for basic and
alanine for neutral or acidic amino acids.

Amino acid	No. of residues/molecule of protein		
	Reported[a]	Found	
		After irradiation (min) of	
		0	240
Lysine	2	1.9	1.9
Histidine	1	0.82	0.0
Arginine	4	(4.0)	(4.0)
Aspartic acid	7	7.0	7.4
Threonine	2	2.1	2.3
Serine	6	5.5	5.6
Glutamic acid	10	10.5	10.4
Proline	9	9.1	9.1
Glycine	15	15.1	15.2
Alanine	9	(9.0)	(9.0)
Cystine	7	7.2	7.0
Valine	3	3.0	3.0
Methionine	0	0.1	0.1
Isoleucine	2	2.0	2.0
Leucine	6	6.2	6.2
Tyrosine	1	0.98	0.06
Phenylalanine	3	3.0	3.2

a) by E. Breslow, et al.[69]

the same molecular weight as native NP-I (Fig. 2).   This shows that NP-I is not subject to
rupture of any peptide bond and that the photodestruction of amino acid side chains has-
occurred as only event on the protein during 240 min of irradiation under the experimental
conditions.

Hormones-Binding Ability of Photooxidized NP-I

To determine the effects of photochemical modification of histidine and tyrosine residues
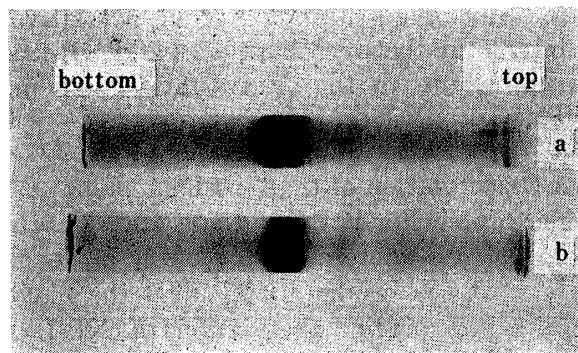of NP-I on the biological activity of the protein, the hormones-binding ability was tested by

Fig. 2. Polyacrylamide Gel Electrophoresis of Photooxidized Neurophysin I

15 % separation gels with 0.1 % sodium dodecyl sulfate and 5 % β-mercaptoethanol.   a, native bovine
neurophysin I; b, irradiated bovine neurophysin I for 240 min in the presence of rose bengal.

pH Hormone Binding Profiles of Photooxidized NP-I

In attempts to elucidate the role of single tyrosine of NP-I, the binding of both oxytocin and [8-arginine]vasopressin to the photooxidized NP-I in which single tyrosine and histidine had been lost was investigated over the pH range 4.0—7.4. As shown in Fig. 4, the pH equilibrium dyalysis. Fig. 3 shows the binding ability of NP-I for oxytocin and vasopressin as a result of photooxidation in the presence of rose bengal. After 5 min of irradiation, at this time only the histidine residue had been completely photooxidized, the hormones-binding ability of the protein was completely retained. This clearly indicates that the single histidine residue of NP-I is unessential in the binding process. After 240 min of irradiation, at this time about 95% of original single tyrosine residue had been photooxidized, the binding ability was decreased to about 40% of original ability. It is of interest that the decrease in binding ability of the modified protein proceeds almost identically for oxytocin and vasopressin as the ligand. These findings suggest that the photochemical modification of single tyrosine residue has some relevance to the decrease in hormones-binding ability of NP-I.
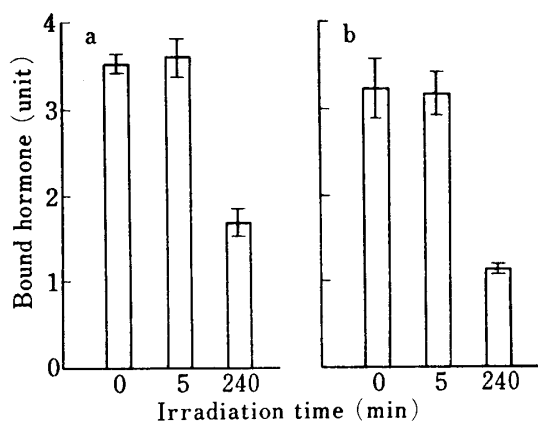

Fig. 3. Hormone-Binding Ability of Neu-
rophysin I Photooxidized in the Presence
of Rose Bengal

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. Column height represents the hormone units bound to 0.5 mg of protein (mean ± S.D., n=3).
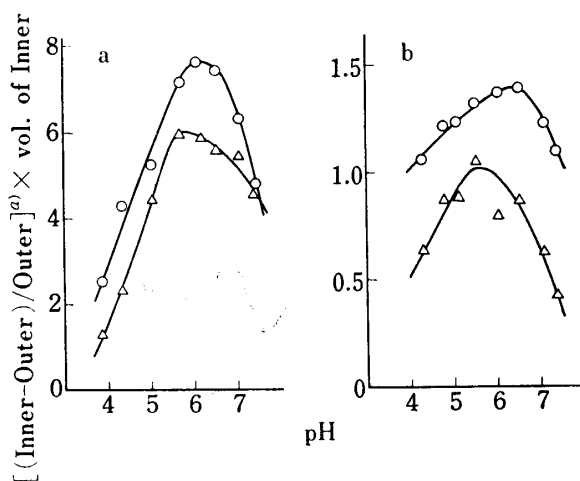a, oxytocin; b, vasopressin

Fig. 4. Binding of Oxytocin (○) and Vaso-
pressin (△) to Native (a) and Photooxidized
(b) Neurophysin I as a Function of pH

a) hormone activity (u/ml) of inner or outer solution in the equilibrium dialysis

binding profiles of photooxidized NP-I for both hormones are found to be essentially identical with those of native protein.   Since the pH binding profiles of native NP-I have been thought to reflect the electrostatic interaction between hormones and NP-I, it seems likely that single tyrosine residue does not contribute to the electrostatic component of the interaction.

Acetylation of NP-I

It has been shown that O-acetylation of the tyrosine residue of NP-II exerts no influence on the binding ability of the protein for both oxytocin and [8-arginine]vasopressin.[6a,9]   To determine the effect of O-acetylation of the tyrosine residue of NP-I on the biological activity of the protein, acetylation of NP-I previously citraconylated was carried out using N-acetylimidazole.   The extent of 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-I produces a characteristic decrease in absorption between 250 and 300 nm (Fig. 5), as expected to O-acetylation of the tyrosine residue.[6a,13]   When the difference in absorbance at 278 nm between O-acetylated and de-O-acetylated proteins, $\Delta\varepsilon_{278} = 1160$ per mole, was used to determine the number of tyrosyl residue modified in the protein[14] 0.99 mole of O-acetyltyrosine was found per 10000 g of protein.   From these results it is evident that the single tyrosine residue of NP-I was acetylated completely under the conditions.
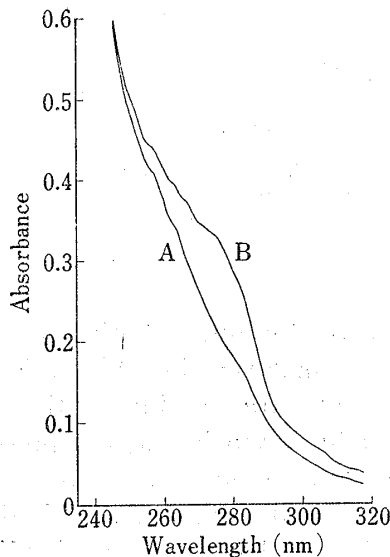

Fig. 5.   Spectra of O-Acetylated (curve A) and De-O-acetylated (curve B) Neurophysin I at pH7.5

The final concentration of protein (0.1 μmol/ml) was identical for the two spectra.

Table II shows the binding abilities of acetyl NP-I and de-O-acetylated protein for both hormones. It was found that the O-acetylation of the tyrosien residue caused no significant changes in the binding ability of NP-I.   This agrees well with the result of the acetylation of NP-II and indicates, together with the results of the pH binding profiles of photooxidized NP-I (see Fig. 4), that the phenolic hydroxyl group of tyrosine-49 of NP-I is not concerned in the binding interaction between NP-I and peptide hormones.

TABLE II.   Effect of Tyrosine O-Acetylation on the Hormones-Binding Ability of Bovine Neurophysin I

Experimental details of acetylation and de-O-acetylation of neurophysin I are as described in the "Experimental."   Hormones-binding abilities were 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.

Neurophysin I	Oxytocin units bound to 0.5 mg of protein[a]	%	Vasopressin units bound to 0.5 mg of protein[a]	%
Native	4.83(±0.15)	100	3.58(±0.20)	100
O-Acetylated	5.17(±0.04)	107.0	3.78(±0.27)	105.6
De-O-acetylated	5.12(±0.19)	106.0	3.56(±0.28)	99.4

a) mean of 3 replicals (±S.D.)

13) J.F. Riordan, W.E.C. Wacker, and B.L. Vallee, Biochemistry, 4, 1758 (1965).
14) J.F. Riordan and B.L. Vallee, "Methods in Enzymology," Vol. 11, ed. by C.H.W. Hirs, Academic Press Inc., New York, 1967, p. 570.

Discussion

The single histidine residue of bovine NP-I could be selectively modified by the irradiation with visible light in the presence of rose bengal (Fig. 1). The photochemical modification of the histidine residue, however, exerted no influence on the binding ability of NP-I for both oxytocin and vasopressin (Fig. 3). The possibility that the histidine residue of NP-I might be unessential in the hormone binding process has been mentioned by Cohen, *et al.* because of the absence of histidine in NP-II and the absence of any significant pK shift for the histidine in the NP-I-oxytocin complex.[6t] Proton magnetic resonance studies of complex formation between NP-I and oxytocin also suggest that lone histidine residue moves to a somewhat more positive (less electron dence) environment in protein but does not play a significant role in the hormonal interaction.[6o] The present study on the photooxidation of the histidine residue of NP-I has provided a direct evidence for the unessential role of the residue of NP-I for hormones-binding.

On the other hand, a decrease in the binding ability of NP-I for both hormones was observed when the tyrosine-49 residue was photooxidized after prolonged irradiation (Table I, Fig. 3). Coupled with the findings that there are no significant photooxidation in other amino acid residues including cystine residues and no evidence for the rupture of peptide bonds of NP-I during irradiation under the conditions, it may be concluded that the decrease in the hormones-binding ability of the photooxidized NP-I is due only to the photodestruction of the tyrosine-49 residue.

Since NP-I and NP-II both possess a very similar amino acid sequence each other[8] and essentially identical affinity constants for either oxytocin or lysine-vasopressin,[7] it may be considered that their hormone binding sites would consist of similar amino acid residues. This may be supported by our findings that tyrosine-49 of both NP-I and II are essential for the hormone binding process of the proteins, while the histidine residue in NP-I and the methionine residue in NP-II, which are residues not in common to the two proteins, are unessential.

It is generally accepted that the hormone-protein interaction involved primarily 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.[6] Walter and Hoffmann have reported preliminary evidence that glutamate-30 or aspartate-31 of NP-II may correspond to the carboxylic side chain which formed an ion pair with NH_2-terminal residue of the ligand.[15] Interestingly, also in NP-I, these amino acid residues were found in position 30 and 31.[8] Furthermore, an intramolecular interaction of such carboxyl group in uncomplexed protein with tyrosine-49 has been implied from spectroscopic studihs.[6d,e] From these previously reported feature on the carboxyl group and our findings that photochemical modification of tyrosine-49 of both NP-I and II similarly resulted in the loss of their hormones-binding ability, it seems likely that the common residues to NP-I and II, glutamate-30 or aspartate-31 and tyrosine-49 may assemble together in spatially close manner and constitute binding site for hormones.

It is of interest to elucidate the fashion of the participation of single tyrosine residue of NP-I in the hormones-binding process. Our previous findings suggested that single tyrosine residue of NP-II might contribute to hormones-binding ability of the protein through its aromatic ring.[9] To examine whether aromaticity of tyrosine-49 is also required in the case of NP-I for its hormones-binding ability, the effect of O-acetylation of tyrosine-49 of NP-I on its binding capacity was investigated. As shown in Table II, there are no significant difference in hormones-binding ability between O-acetylated and de-O-acetylated protein.

15) R. Walter and P.L. Hoffmann, *Fed. Proc.*, **32**, 567 (1973).

This suggests that the tyrosine residue of NP-I may also contribute to hormones-binding ability of the protein through its aromatic ring since the aromatic ring has been believed to be cleaved in the dye-sensitized photooxidation of tyrosine,[16] while the aromatic ring has been still retained after O-acetylation of tyrosine. Such common requirement of the aromaticity of tyrosine-49 in both NP-I and II for their hormones-binding ability, together with the spectroscopic observations that upon complex formation with both hormones the common residue, tyrosine-49, moves similarly from a less to more polar environment in both proteins,[6d–f,j,k,17] may suggest a common role of the tyrosine-49 for the hormone binding in NP-I and NP-II. We have already presumed that, in NP-II, a positive cooperativity in binding may exist between tyrosine-49 and the carboxyl group which serve as an electrostatic constituent for binding and this may provide the principal driving force for hormone binding; the destruction of aromaticity of tyrosine-49 results in the loss of the cooperative factor of the tyrosine and nearby carboxyl group which existed in native protein and this may lead to weaking of binding ability of NP-II.[9] The role of the tyrosine-49 residue of NP-I may also be explained as the same conception as described for NP-II.

Recently, Wolff, et al. have indicated that nitration of tyrosine-49 modifies the stoichiometry of the NP-[8-lysine]vasopressin reaction and results in a slight increase in the affinity of oxytocin for the protein without any change in the stoichiometry.[17] On the other hand, Furth and Hope indicated that the [8-arginine]vasopressin binding ability of NP-II was found to be unchanged by nitration of the tyrosine residue.[6d] Though the molecular basis for the difference in behavior of oxytocin versus vasopressin is not yet clear, it seems most probable that nitration of the tyrosine-49 induces some subtle conformational rearrangements which may be responsible for the different effect on the binding ability of the protein for oxytocin and two kinds of vasopressin. The case of the photodestruction of the tyrosine-49 of NP-I as well as NP-II, however, led to almost identical loss of its binding ability for both oxytocin and vasopressin. This may provide the evidence for the direct participation and similar contribution of the aromatic ring of tyrosine-49 of both NP-I and II in binding process with both hormones rather than the possibility that photochemical modification of the tyrosine-49 induces the conformational changes of the protein as a primary event and thereby affects on binding ability of the proteins. This may also be drawn from findings that the binding of both hormones to NP-I or NP-II is accompanied similarly by perturbations of the tyrosine-49 of the proteins.[6d–f,17]

It has been suggested that within certain secretory neurons of the hypothalamo-neuro-hypophysial system the bovine NP-I is bound in vesicles with oxytocin and NP-II with vasopressin,[5] whereas both isolated NP-I and NP-II can bind both hormones with essentially identical affinity.[7] This specific location of NP and hormones in neurosecretory granules has been thought simply as a result of compartmentalization during the biosynthesis of the hormones and NP proteins.[7] An object of a series of our study on the selective chemical modification of special amino acid residues of isolated NP-I and NP-II is to elucidate whether or not more specific molecular basis of the association between NP and neurohypophysial hormones are existed. If some information by which one hormone is determined to bind specifically to one protein is assigned on the amino acid sequences of the protein, modification of the corresponding amino acid residues would be expected to result in some changes in hormone-binding specificity of the protein. In the limitation of our studies, however, any significant change in specificity of NP-I or NP-II could not be found. Although the single histidine residue in NP-I and methionine residue in NP-II are characteristic residue of each protein, photochemical modification of these specific residues give rise to no significant change in

16) 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).

17) J. Wolff, R. Alazard, M. Camier, J.H. Griffin, and P. Cohen, J. Biol. Chem., 250, 5215 (1975).

binding ability of each protein to each hormone (Fig. 3).[9] Therefore, it seems unlikely that lone histidine of NP-I and lone methionine of NP-II are direct determinants of the binding specificity of each protein to oxytocin and vasopressin, respectively. The possibility that some other amino acid residue on NP-I or NP-II may serve as the determinants of the hormone binding specificity of the protein has still been remained to be elucidate.