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Chemical Modification and Cross-Linking of Neurophysin Tyrosine-49†

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ABSTRACT: Photoaffinity labeling of the single neurophysin tyrosine, Tyr-49, with Met-Tyr-azido-Phe amide has been reported to inhibit both neurophysin self-association and peptide binding. Accordingly, we investigated the functional consequences of modification, principally by tetranitromethane, of Tyr-49. Tetranitromethane-mediated tyrosine–tyrosine cross-linking permitted synthesis of covalent neurophysin "dimers" and of peptide–protein conjugates, the latter potentially analogous to the photoaffinity-labeled product. The self-association and binding properties of the covalent dimers were found to be similar or enhanced relative to those of the native protein. In contrast to the photoaffinity-labeled product, covalent conjugates of Tyr-49 with the ligand peptides Met-Phe-Tyr amide, Phe-Tyr amide, and Tyr-Phe amide also generally exhibited normal or increased binding affinity for exogenous peptide; a subfraction of the Phe-Tyr amide adducts showed evidence of reduced affinity. Diiodination of Tyr-49 had no significant effect on binding. However, among the products of tetranitromethane treatment in the absence of peptide was a novel inactive non-cross-linked product, representing modification only of Tyr-49 but containing no demonstrable nitrophenol. As evidenced by circular dichroism and nuclear magnetic resonance (NMR), this product was not significantly unfolded and retained the ability to self-associate. These latter results provide the strongest evidence thus far of a role for Tyr-49 in peptide–hormone binding. The disparate effects of different Tyr-49 modifications are collectively interpreted and reconciled with NMR data and the properties of the photoaffinity-labeled protein to suggest potential mechanisms of Tyr-49 participation in binding and the probable orientation of Tyr-49 relative to peptide residue 3 in neurophysin complexes.

The posterior pituitary protein neurophysin binds the peptide hormones oxytocin and vasopressin within the hypothalmo–neurohypophyseal tract and interacts similarly with di- and tripeptides resembling the amino-terminal region of the hormones (Breslow, 1979; Cohen et al., 1979). The binding site common to these peptides is largely unidentified.[1] A central question has been the relative contribution to binding of nonduplicated and internally duplicated regions of the protein [e.g., see Breslow (1979) and Cohen et al. (1979)]. In this context, the single neurophysin tyrosine, Tyr-49, of the evolutionarily conserved nonduplicated region has been extensively

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[1] The binding site investigated in this study is the principal hormone site, to which the smaller peptides also bind. A second site, which may also bind the hormones under some conditions, but which is not of thermodynamic significance for peptides, may also be present and close to Tyr-49 [e.g., see Cohen et al. (1979) and Breslow (1984)].

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investigated.[1] Tyr-49 is perturbed by the binding of peptides [e.g., see Furth & Hope (1970) and Breslow & Weis (1972)], although the ring appears to have fewer motional constraints and greater accessibility to solvent in the bound than in the free state (Balaram et al., 1972; Griffin et al., 1973). Mononitration or acetylation of Tyr-49 is without effect on binding activity (Furth & Hope, 1970), and dinitration is associated with only a small activity decrease (Breslow & Gargiulo, 1977). Photooxidation has been reported to decrease activity (Fukuda et al., 1976a,b), but the magnitude of the effect is unclear. Nuclear magnetic resonance (NMR)[2] distance measurements allow a distance between the Tyr-49 ring and the side chain of residue 1 of bound peptides of less than 10 Å but suggest significantly longer distances (≥13 Å) between Tyr-49 ring protons and the side chain of peptide residue 3 (Lord & Breslow, 1980; Peyton & Breslow, 1985; Peyton et al., 1987).

Recently, Abercrombie et al. (1982, 1984) used the peptide Met-Tyr-azido-Phe-NH_2 to photoaffinity label the binding site and isolated a product, apparently labeled only at Tyr-49, that had lost the ability both to bind added peptide and to self-associate. However, identification of Tyr-49 as the site of peptide attachment was indirect, and the loss of self-association was unexpected. Additionally, these studies suggested a closer proximity of Tyr-49 to residue 3 of bound peptides than suggested by NMR. Accordingly, we set out to examine the functional effects of other modifications of Tyr-49, specifically iodination and reaction with tetranitromethane (TNM). The nitrated products of reaction of neurophysin with tetranitromethane have been previously investigated [e.g., see Furth & Hope (1970) and Breslow & Gargiulo (1977)]. However, TNM treatment of phenols generates other products, including oxidatively cross-linked phenols [e.g., see Bruice et al. (1968), Williams & Lowe (1971), Aeschbach et al. (1976), and Verweij et al. (1982)], and therefore provides the opportunity to specifically link Tyr-49 either to another Tyr-49 or to the tyrosine of ligand peptides. If Tyr-49 is close enough to the binding site such that its reaction with a photoaffinity label blocks binding, some of these cross-linked products might similarly be expected to be functionally inactive. The products of cross-linking of neurophysin to itself and to three ligand peptides, each representing the placement of tyrosine at a different position at the binding site, were studied. The ligand peptides were Met-Phe-Tyr-NH_2 (potentially analogous to the photoaffinity label), Phe-Tyr-NH_2, and Tyr-Phe-NH_2; note that the α-NH_3^+ terminus defines the placement of the peptide in the binding site (Breslow, 1979). Additionally, a novel non-cross-linked product of nitration was obtained and partially characterized. The properties of these products provide new insights into the relationship of Tyr-49 to the peptide binding site.

Materials and Methods

Preparation of Native and Modified Neurophysins. Bovine neurophysins I and II were prepared as previously described (Breslow et al., 1971). Iodination was performed by using a modification of the method of Morrison and Bayse (1970). Neurophysin II (30 mg) was dissolved in 12.5 mL of phosphate–EDTA buffer (0.05 M phosphate, pH 7.4, and 10^{-3} M EDTA). Lactoperoxidase (0.42 mg total, Sigma) was added in small additions to the stirring protein solution over a period of 40 min, together with small additions of KI (0.75 mg total) and H_2O_2 (0.36 mg total). The final solution was stirred for an additional hour, concentrated, and chromatographed on Sephadex G-50 in 0.1 M acetic acid. The principal protein peak was lyophilized and used for the studies reported.

For studies of TNM reaction products, neurophysin II was additionally purified by affinity chromatography (Rabbani et al., 1982). The protein (2×10^{-4} M) was then treated for 2 h with TNM at pH 8.1 as previously described (Furth & Hope, 1970; Breslow & Weis, 1972) with additions and modifications noted under Results. For TNM reaction in the presence of peptide, limited peptide solubility at pH 8.1 necessitated carrying out some reactions for 24 h at pH 6.8 (Results). Control studies of products formed at pH 6.8 and 8.1, in cases where the same reaction could be monitored at both pH values, indicated that the rate of reaction, but not the distribution of products, was affected by the pH difference. For studies in which the relative yields of different products under different conditions were to be compared, the same starting lot and concentration of affinity-purified neurophysin were used. Following reaction with TNM, the reaction mixture was chromatographed on Sephadex G-25 in 0.1 M acetic acid and the protein peak lyophilized. The protein was resolved into binding and nonbinding components by affinity chromatography (Rabbani et al., 1982), and the different components were fractionated on Sephadex G-50 or G-75 in 0.1 M acetic acid or 1 M formic acid. In some studies, fractionation by size preceded affinity chromatography.

Determination of Binding Affinity. Two spectroscopic methods, CD and fluorescence, were used. CD studies involved monitoring deviations from spectral additivity at 278 nm associated with addition of Phe-Tyr-NH_2 to the protein at pH 6.1 in 0.16 M KCl–0.005 M citrate. A computerized Jobin-Yvon Mark 5 spectrometer was used. For each addition of peptide, the "theoretical" sum of the spectra of protein and added peptide was computed from the spectra of the individual components. This spectrum was subtracted from the observed spectrum to give the binding-induced spectral changes. The magnitude of these changes at each peptide addition relative to those representing complete binding (one peptide per polypeptide chain) was used to calculate the degree of protein saturation and the concentration of unbound peptide, as described previously for CD studies of nitrated neurophysin (Breslow et al., 1973). Fluorescence binding studies were performed on a Perkin-Elmer MPF-4 spectrofluorometer as described previously (Sur et al., 1979) except that excitation was at 352 nm and emission was monitored at 410 nm (Results).

Binding affinities were also determined by affinity chromatography, using a dipeptide-linked affinity column (Rabbani et al., 1982). Under standard conditions, protein is applied to the column at pH 6.2, elution continued for 30 mL, and the pH then changed to 2.1. Inactive protein elutes at the void volume (7 mL) at pH 6.2, and native protein elutes at the void volume at pH 2.1 due to decreased binding affinity at low pH. In this study, the column was additionally calibrated for intermediate binding affinities by determining the elution volume of the mononitrated protein at several pH values and comparing this volume with the known effect of pH [e.g., see

[2] Abbreviations: CD, circular dichroism; NMR, nuclear magnetic resonance; TNM, tetranitromethane; Phe-Tyr, L-phenylalanyl-L-tyrosine; Phe-Tyr-NH_2, L-phenylalanyl-L-tyrosine amide; Phe-Phe-NH_2, L-phenylalanyl-L-phenylalanine amide; Tyr-Phe-NH_2, L-tyrosyl-L-phenylalanine amide; Met-Phe-Tyr-NH_2, L-methionyl-L-phenylalanyl-L-tyrosine amide; Met-Tyr-azido-Phe-NH_2, L-methionyl-L-tyrosyl-L-4-azidophenylalanine amide; Boc-Met-Phe, *tert*-butyloxycarbonyl-L-methionyl-L-phenylalanine; Tyr-NH_2, L-tyrosine amide; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; EDTA, ethylenediaminetetraacetic acid; NP-II, neurophysin II.

Breslow & Gargiulo (1977)] on binding affinity. The results indicated that protein eluting in the void volume at pH 6.2 has an affinity ≤0.01 that of the native protein, while protein not eluting until after the pH is lowered to 2.1 has an affinity at least 0.1 that of the native protein.

For peptide-coupled samples that were insoluble at pH 6, affinity chromatography was also performed with an initial elution pH near 3, followed by pH 2.1. Although neurophysin binds peptide more weakly at pH 3 than at pH 6, it is sufficiently retarded at pH 3 to permit functional differences among protein samples to be assessed under these conditions (Results).

Peptides. Met-Phe-Tyr-NH_2 was synthesized by coupling 1-hydroxybenzotriazole-activated Boc-Met-Phe (Vega) to Tyr-NH_2 (Vega) in tetrahydrofuran; the Boc group was removed by using HCl in ethyl acetate. The final product moved as a single spot (visualized by I_2, ninhydrin, and UV quenching) on silica gel thin-layer chromatography using $CHCl_3/CH_3OH$ (6:1) and 1-butanol/acetic acid/H_2O (4:1:1) as mobile phases. Amino acid analysis of the final product gave a Met:Phe:Tyr ratio of 0.93:1:1; comparison of the UV spectrum with that expected for Met-Phe-Tyr-NH_2·HCl indicated >99% purity. The peptide Tyr-Phe-NH_2 was a gift from Dr. John Rose of the Biocrystallography Laboratory at the Veterans Administration Hospital in Pittsburgh. Phe-Tyr was obtained from Vega. Other peptides are those reported previously (Sur et al., 1979; Breslow et al., 1973).

The binding constant at 25 °C, pH 6.2 for Phe-Tyr-NH_2 or Phe-Phe-NH_2 (1.2×10^4 M^{-1}) is that previously reported (Breslow et al., 1973; Whittaker et al., 1985). Binding constants for Phe-Tyr, Tyr-Phe-NH_2, and Met-Phe-Tyr-NH_2 were determined by CD (manuscript in preparation); values obtained were 3×10^2, 5×10^4, and 5×10^3 M^{-1}, respectively.

HPLC. HPLC was performed by using a 0.46 × 25 cm Du Pont CN reverse-phase column (Abercrombie et al., 1982) on an IBM LC/9533 equipped with a variable wavelength detector and integrator. The solvent gradient represented a change from solvent A (80% 0.25 M triethylammonium phosphate, pH 3.0/20% acetonitrile) to solvent B (70% 0.25 M triethylammonium phosphate, pH 3.0/30% acetonitrile) in 20 min and was then held at solvent B for 15 min. Under these conditions, affinity-purified neurophysin II eluted at 21 min with a 1.2-min period from the beginning of the slightly asymmetrical peak to its completion, the asymmetry reflecting a typically contaminating minor neurophysin (Rabbani et al., 1982).

Other Methods. NMR studies were performed as described elsewhere (Peyton et al., 1986). Analytical ultracentrifuge studies were carried out at 52 000 rpm in double-sector cells on a Model E Beckman ultracentrifuge equipped with a UV scanner. Absorbance was monitored at 280 nm; buffer, against which the protein had been dialyzed, was used as the reference. Polyacrylamide gel electrophoresis in the absence of SDS (standard PAGE) or in the presence of SDS and mercaptoethanol was performed as previously described (Rabbani et al., 1982). Amino acid analyses were also carried out as described previously (Rabbani et al., 1982) with the modification that 10^{-4} M phenol was added to some samples to protect tyrosine during acid hydrolysis so as to ensure that the apparent absence of tyrosine in these fractions was not an artifact; amino acid analyses were identical in the absence and presence of the added phenol.

RESULTS

Properties of Iodinated Neurophysin II. On standard PAGE at pH 9.5, the iodinated product migrated as a single band, faster than the native protein. Spectrophotometric titration between pH 5 and 11 at 260, 305, and 325 nm demonstrated a single titratable phenolic species with $pK_a = 7.1$ (data not shown). Comparison with pK_a values of 8.2 and 6.4 for monoiodotyrosine and diiodotyrosine, respectively [e.g., see Edelhoch (1962)], utilizing the fact that the pK_a of Tyr-49 is 0.6–0.7 pH unit above "normal" because of a neighboring carboxylate [e.g., see Breslow & Weis (1972)], placed the observed pK_a in good agreement with that expected for diiodinated Tyr-49. Additionally, the 314-nm location of the near-UV absorption maximum at pH 10 (data not shown) compared with values of 305 and 311 nm for the ionized forms of monoiodotyrosine and diiodotyrosine, respectively (Edelhoch, 1962), further identifying the final product as the diiodinated derivative. The affinity of the iodinated product for Phe-Tyr-NH_2 was measured by CD (Materials and Methods). Scatchard analysis of the data (Figure 1) demonstrated a binding constant of (8–9) × 10^3 M^{-1} which compares with values under similar conditions of ~1.2×10^4 M^{-1} for mononitrated neurophysin II (Breslow & Gargiulo, 1977) and ~7×10^3 M^{-1} for native neurophysin I (Lundt, 1977).

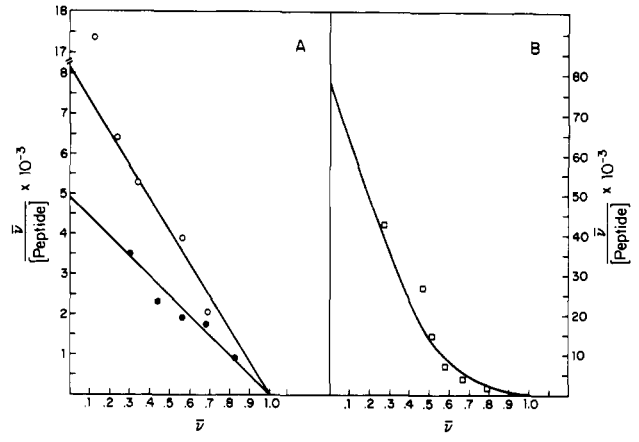

FIGURE 1: Scatchard plots of peptide binding to modified neurophysins, pH 6. (A) Binding of Phe-Tyr-NH_2 to iodinated neurophysin (10^{-4} M) as measured by CD (○); binding of Phe-Phe-NH_2 to the high molecular weight component (5×10^{-5} M) from TNM treatment as measured by fluorescence (●). (B) Binding of Phe-Tyr-NH_2 to the high molecular weight component (5×10^{-5} M) as measured by CD (□). The solid line in this case is theoretical for a two-component system in which half of the material has a binding constant of 1.5×10^5 M^{-1} and half has a binding constant of 5×10^3 M^{-1}.

Products of Reaction of Neurophysin II with Tetranitromethane. Under conventional conditions (Furth & Hope, 1970), the principal product of the reaction of neurophysin II with TNM is mononitrated at Tyr-49 and has a completely functional peptide binding site. Of the minor products previously detected, one is dinitrated at Tyr-49 with half the binding affinity of the mononitrated protein (Breslow & Gargiulo, 1977), and another has a high molecular weight (Wolff et al., 1975) but has not otherwise been characterized. We further characterized the minor products of TNM treatment by affinity and gel filtration chromatography. When affinity chromatography purified neurophysin II is treated with TNM and resubjected to affinity chromatography, a significant fraction (~17% by weight, 28% by Coomassie stain color) is not retarded by the column (Figure 2). Control studies indicate that this inactive product is not formed by any of the nonspecific procedures (gel filtration, lyophilization, etc.) used in workup of the TNM product. When the active product from the affinity column is chromatographed on Sephadex G-50, two components are seen (Figure 2). The principal product (peak II) is largely mononitrated neurophysin II, as judged by amino acid analysis (Table I), but can contain traces

Table I: Amino Acid Compositions of Native and Tetranitromethane-Treated Bovine Neurophysin II Fractions

	native protein[a]	fractions from initial TNM treatment of NP-II			nonbinding fraction from "renitration"
		nonbinding	high mol wt (binding)	mononitrated (binding)	
Asp	5.0 (5)	5.1	4.8	4.7	5.3
Thr	2.0 (2)	2.0	1.9	1.8	2.0
Ser[b]	5.9 (6)	5.7	5.3	4.9	5.9
Glu	13.0 (13)	13.0	13.0	12.0	13.0
Pro[c]	9.4 (8)	ND	ND	6.7	ND
Gly	14–16 (15)	15.0	15.0	14.0	15.0
Ala	6.3 (6)	5.6	5.8	6.1	6.0
$^1/_2$-Cys[c]	13.0 (14)	13.0	10.0	13.0	10.0
Val	3.8 (3.7)	3.8	3.8	3.9	3.4
Met	1.0 (1)	0.8	0.9	1.0	0.7
Ile	2.4 (2.3)	2.3	2.4	2.4	2.0
Leu	6.0 (6)	6.2	6.0	6.2	5.8
Tyr	1.0 (1)	0.1	0	0	0
NO_2-Tyr	0 (0)	0	0	1.1	0
Phe	3.1 (3)	3.0	2.8	3.1	2.7
His	0 (0)	0	0	0	0
Lys	2.0 (2)	2.0	2.0	2.0	2.0
Arg	7.0 (7)	7.0	7.0	7.3	6.5

[a] Values in parentheses are those calculated from sequence data [e.g., see Breslow (1979)]; nonintegral values reflect demonstrated heterogeneity at position 89. Values are normalized to two Lys per chain. [b] Serine values are uncorrected for loss during hydrolysis (0–20%). [c] Proline and half-cystine values are unreliable and were often not determined (ND).

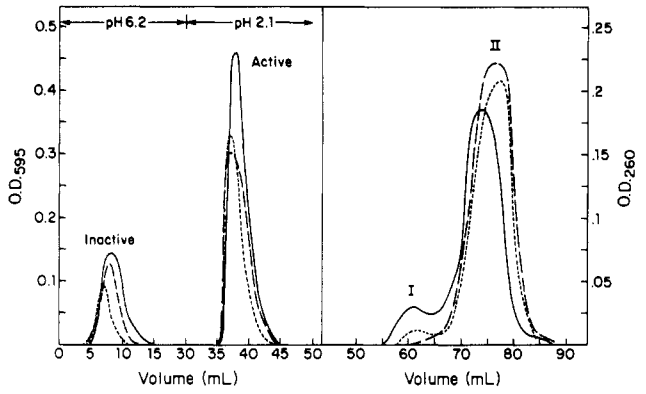

FIGURE 2: Chromatographic behavior of neurophysin components from TNM treatment at pH 8. (Left) Affinity chromatography of products from treatment of native protein alone (—), native protein in the presence of 10^{-3} M Phe-Tyr (···), and mononitrated protein (---). Protein concentration is monitored with Coomassie Blue G-250 (Materials and Methods). (Right) Sephadex G-50 chromatography (1.6 × 70 cm column) of the active (binding) components obtained from affinity chromatography; key is the same as on the left. Protein concentration is monitored by UV absorbance. Peak I, where present, is the high molecular weight product. Peak II is nitrated neurophysin.

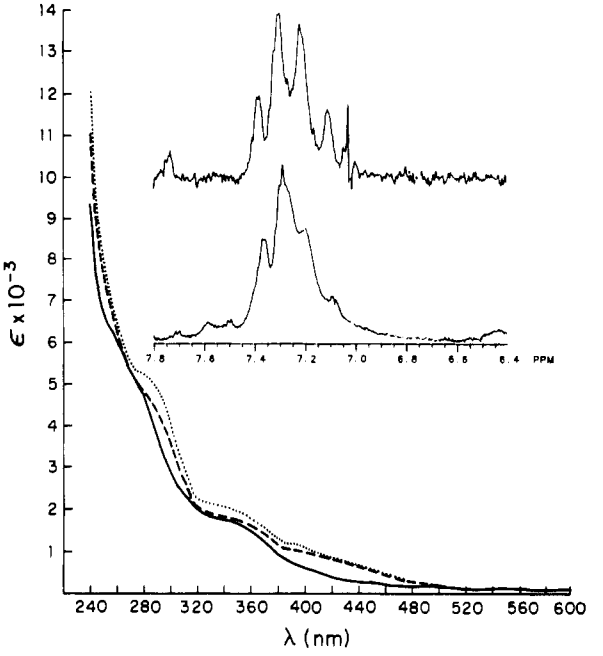

FIGURE 3: Spectral properties of the inactive product from TNM treatment. (Main figure) Absorption spectra of the inactive neurophysin II product at pH 6 (—), pH 9 (---), and pH 10.3 (···). (Inset) 500-MHz proton NMR spectra of the inactive neurophysin I product at pH 6 in D_2O. Top spectrum, 2×10^{-5} M; lower spectrum, 7.5×10^{-4} M. Chemical shifts are in ppm downfield from sodium 3-(trimethylsilyl)propionate-d_4.

of the dinitrated product (Breslow & Gargiulo, 1977). The minor G-50 product (peak I) emerges before the main fractions; i.e., it behaves as if it has a higher molecular weight.

Amino acid compositions of the products of TNM treatment (Table I) indicate that the inactive product and the active high molecular weight product contain neither tyrosine nor nitrotyrosine; other amino acids are the same as in the mononitrated protein. Spectrophotometric titration of these products, as described elsewhere (Breslow & Gargiulo, 1977), also indicated the absence of dinitrotyrosine. Further characterization of each is given below.

Properties of the Inactive Tetranitromethane Product. Elution of the inactive product in or just after the void volume (7 mL) of the affinity column indicates an affinity ~0.01 that of native neurophysin. On SDS gel electrophoresis in the presence of mercaptoethanol, the inactive product gives a single band of molecular weight ~10000, the same as native protein (data not shown). However, on standard PAGE, three intense bands are seen, together with a faint smear indicative of grossly denatured neurophysin (data not shown); the mobilities of the products indicate the addition of zero, one, and two negative charges, respectively, to the native protein. When affinity-purified *mononitrated* neurophysin was retreated with TNM, inactive product, again marked solely by a lack of either tyrosine or nitrotyrosine (Table I), was again produced, in comparable yield to that obtained from native neurophysin (Figure 2). Similar inactive products were obtained from nitration of both bovine neurophysin II and neurophysin I.

Visible and UV absorption studies of the inactive product (Figure 3) demonstrated significant absorption near 350 nm, suggesting the presence of a nitro group. However, increasing the pH from 6 to 10 (Figure 3), or to pH 13 (not shown), had only modest effects on the spectrum in the 350–500-nm region, indicating the absence of significant nitrophenol content. Thus, the observed $\Delta\epsilon$ with pH in the 440-nm region can be com-

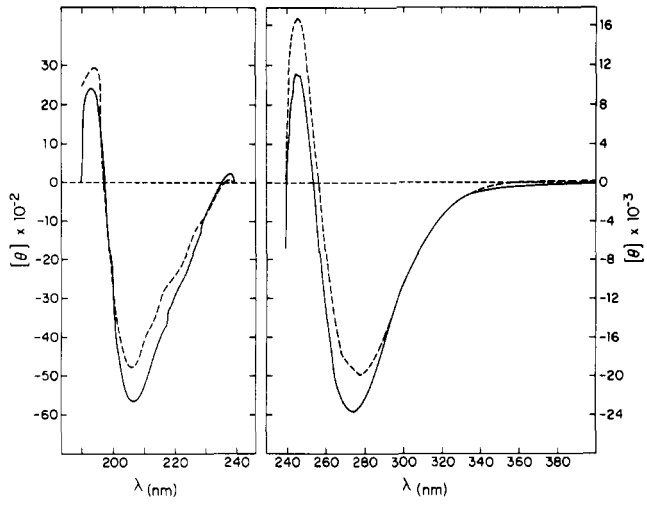

FIGURE 4: CD spectra of native bovine neurophysin II (---) and of the inactive component from TNM treatment (—), pH 6. (Left) Far-UV spectra, plotted as residue ellipticities (in degrees centimeter squared per decimole). (Right) Near-UV spectra plotted as molar elipticities (in degrees centimeter squared per decimole).

pared with a $\Delta\epsilon_{440}$ value of ~2700 associated with the ionization of mononitrated neurophysin (Breslow & Weis, 1972). An aromatic proton NMR study of the inactive derivative produced by TNM treatment of neurophysin I is also shown in Figure 3. At pH 6, the data indicate the total absence of a signal at or near 6.78 ppm, the characteristic position of Tyr-49 ring ortho protons in the native protein (Sardana & Breslow, 1984). The results clearly demonstrate that the Tyr-49 ring has been modified in the inactive product. Increasing the pH to 9.5 had no effect on the aromatic region (apart from changes in His protons), again demonstrating the absence of a simple nitrophenol (data not shown). The results demonstrate an unusual modification of the Tyr-49 ring.

CD studies (Figure 4) demonstrated differences between the inactive and native proteins, but these were distinctly different from those associated (Menendez-Botet & Breslow, 1975) with gross unfolding (e.g., total collapse of the 245-nm band and markedly diminished 280-nm negative ellipticity) and may reflect the altered Tyr-49 chromophore. Retention of native conformation in the inactive product was also demonstrated by NMR. In Figure 3, large effects of concentration on the NMR spectrum of the inactive product are seen. These include a large decrease in intensity of a band at 7.22 ppm and increased resolution of a band at 7.57 ppm with increasing concentration. These concentration-dependent changes are identical with those in the native protein (Peyton et al., 1986) and demonstrate that the inactive product retains the ability to self-associate. It is relevant that, in the inactive product, a band near 7.1 ppm is also concentration dependent; this may represent a proton derived from Tyr-49, since Tyr-49 spectra are also concentration dependent in the native protein. Comparison of the aliphatic proton region of native neurophysin and its inactive derivative at 0.7 mM concentration indicated no obvious differences (data not shown).

Characterization of the Active High Molecular Weight Tetranitromethane Product. In contrast to native neurophysin, the high molecular weight fraction had an apparent molecular weight of ~21 000 on SDS gel electrophoresis (data not shown), indicating a covalently linked dimer. On standard PAGE at pH 9.5, two closely spaced components of equal intensity were resolved (data not shown).

The dimeric nature of the high molecular weight products and their lack of tyrosine or nitrotyrosine argued that each

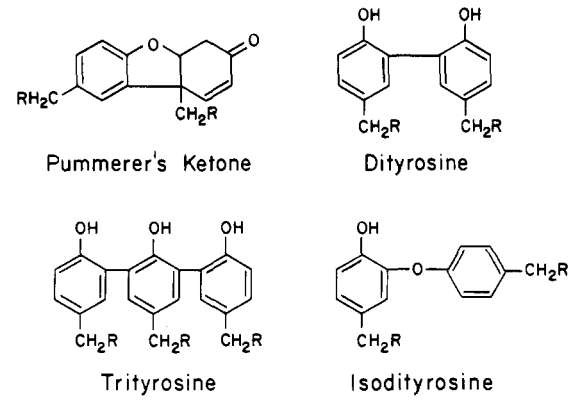

FIGURE 5: Potential products of tyrosine–tyrosine cross-linking by TNM. In the present case, R = tyrosine α-carbon and the rest of the protein chain.

component represented two neurophysin chains cross-linked through their single tyrosines, in keeping with the expected action of TNM on tyrosine-containing compounds (see the introduction). Different cross-linked products are possible [e.g., see Williams & Lowe (1971)] as shown in Figure 5. From the proposed free radical mechanism for formation of such products (Bruice et al., 1968), they should be formed less readily from nitrotyrosine than from tyrosine since nitrotyrosine is less able to form the appropriate radical. Accordingly, we studied the effect of TNM on previously mononitrated monomeric neurophysin (peak II in Figure 2); no high molecular weight products were formed from the previously nitrated product (Figure 2). Additionally, formation of the high molecular weight product from native neurophysin was reduced by 60% if Phe-Tyr, which does not significantly bind to neurophysin under these conditions,[3] was added during reaction with TNM (Figure 2). This is the expected result of competition by the peptide tyrosine for formation of tyrosine–tyrosine cross-links with the protein. No reduction of dimer formation occurred in the presence of Phe-Phe-NH_2 which cannot compete for cross-link formation (data not shown).

The above results indicate that the dimeric products are formed by tyrosine–tyrosine cross-linking, the two products presumably representing different cross-links. The presence of a small amount of dityrosine or a closely related product was indicated by the fluorescence near 410 nm upon excitation at 352 nm (Figure 6). Evidence of a nitrated analogue of isodityrosine was also obtained (data not shown).

Self-Association and Peptide Binding Properties of the High Molecular Weight Product. At pH 6.2, the average sedimentation coefficient ($s_{20,w}$) of the covalent dimers at 1.2 mg/mL was 3.4 S, with scanner patterns displaying markedly broadened boundaries indicative of heterogeneity (data not shown). The observed $s_{20,w}$ is higher than the value of 2.2 S found for the noncovalent neurophysin dimer in the absence of ligands (Rholam & Nicolas, 1981) or the theoretical maximum of 2.9 S calculated for a spherical unhydrated dimer. This fact and the marked broadening of the sedimentation pattern indicate that the covalently cross-linked dimer self-associates, the bivalent nature of each dimer presumably allowing a complex mixture of noncovalent oligomers to be formed. In the presence of saturating concentrations of Phe-Phe-NH_2, the average $s_{20,w}$ value of the cross-linked

[3] The binding constant of Phe-Tyr to mononitrated neurophysin II (2 mg/mL) is 3×10^2 M^{-1} at pH 6.2 and 84 M^{-1} at pH 8, too low for significant binding at pH 8, 10^{-3} M peptide.

Table II: Partial Amino Acid Compositions of "Monomeric" Fractions of Peptide-Coupled Neurophysin II Separated by Affinity Chromatography at pH 3

	Met-Phe-Tyr-NH_2 products		Phe-Tyr-NH_2 products		Tyr-Phe-NH_2 products	
amino acid[a]	retarded[b]	nonretarded	retarded	nonretarded	retarded	nonretarded
Met (1)	2.1 ± 0	2.2	1.2	1.2	1.1	1.4
Leu (6)	6.1 ± 0.1	5.8	5.9	6.0	6.5	6.7
Tyr (1)	[c]	[c]	0.17	[c]	[c]	[c]
NO_2-Tyr (0)	0.27 ± 0.1[d]	[c]	[c]	[c]	0.30[d]	0.38[d]
Phe (3)	4.3 ± 0.1	4.2	5.1	4.7	5.3	5.2
Lys (2)	2.0 ■ 0	2.0	2.0	2.0	2.0	2.0

[a] Values are shown for Met, Tyr, NO_2-Tyr, and Phe for comparison with Lys and Leu. Other amino acids (in addition to Lys and Leu) were unchanged from normal values. Values are given as residues per chain normalized to Lys = 2. Values in parentheses are those in the native protein. [b] Average of two different preparations. [c] Trace value; quantity was too small to permit integration. [d] The nitrotyrosine content in these fractions appears to represent contaminating mononitrated protein (see text footnote 4).

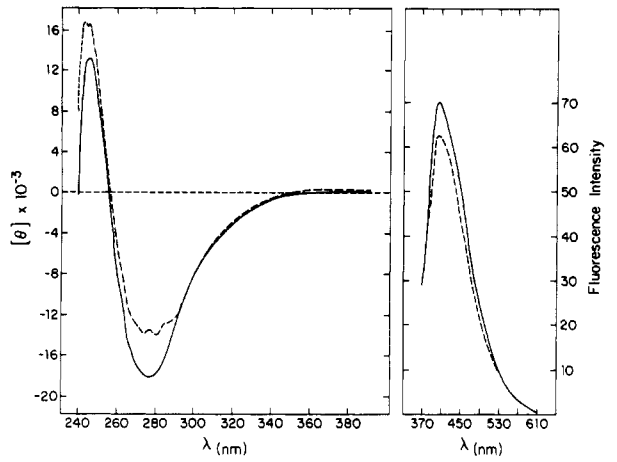

FIGURE 6: Spectral effects of peptides on the high molecular weight product from TNM treatment. (Left) Near-ultraviolet CD spectrum of the high molecular weight product (5×10^{-5} M) alone (—) and in the presence of 5×10^{-4} M Phe-Tyr-NH_2 (---). Results are plotted as protein molar ellipticities. The spectrum in the presence of peptide represents the observed spectrum of the mixture from which the contribution of added peptide has been subtracted. (Right) Fluorescence emission spectra of the high molecular weight product (5×10^{-5} M) obtained on excitation at 352 nm: (—) protein alone; (---) protein plus 10^{-3} M Phe-Phe-NH_2. Intensities are in arbitrary units.

protein increased to 4.7 S (data not shown), indicating an increased degree of oligomerization and/or a decreased frictional ratio, both of which are associated with peptide binding to the native protein (Breslow et al., 1973; Cohen et al., 1979; Rholam et al., 1982).

The peptide affinity of the cross-linked dimer is ≥0.1 that of the native protein since it is retained by the affinity column at pH 6.2 (Materials and Methods). More precise affinities were obtained spectroscopically. In the presence of high concentrations of Phe-Phe-NH_2, the fluorescence intensity of the dityrosine neurophysin component decreased by 10% (Figure 6). Fluorescence titration with Phe-Phe-NH_2 gave a binding constant of $\sim 5 \times 10^3$ M^{-1} (Figure 1) which compared with $\sim 1 \times 10^4$ M^{-1} for peptide binding to native neurophysin under similar conditions (Sur et al., 1979). CD studies of Phe-Tyr-NH_2 binding to the cross-linked dimer (Figure 6) demonstrated ellipticity changes qualitatively and quantitatively analogous to those seen with native neurophysin (Breslow & Weis, 1972), suggesting that both chains of the dimer bind peptide. Titration of these changes indicated two binding site populations. Figure 1 compares the Scatchard data derived from the CD titrations with a theoretical curve calculated by assuming that 50% of the high molecular weight product had a binding constant of 1.5×10^5 M^{-1} (>10 times the normal affinity) and the remaining 50% had a binding constant, as determined by fluorescence, of 5×10^3 M^{-1} (half the normal affinity). If the fluorescence data were ignored, the independently estimated weaker binding constant from CD alone was 3×10^3 M^{-1}.

Cross-Linking of Neurophysin to Ligand Peptides: General Features of the Reaction. Cross-linking of Tyr-49 to the tyrosine of Phe-Tyr-NH_2, Tyr-Phe-NH_2, and Met-Phe-Tyr-NH_2 was achieved by treatment with TNM in the presence of 10^{-3} M peptide. Cross-linking of Phe-Tyr-NH_2 was carried out at both pH 8.1 and pH 6.8 with identical results (Materials and Methods). The other peptides were cross-linked at pH 6.8 because of peptide insolubility at pH 8. At pH 6.8, the estimated degree of protein saturation by bound peptide is 80–95%.

The TNM reaction in the presence of each of these peptides differed from that under other conditions by formation of a protein product that was insoluble at pH 6.2 and represented approximately 25% of the initial protein. This was absent (≤1% of total protein) if the starting protein in the reaction was the previously nitrated protein or if the peptide used contained no tyrosine. Amino acid analyses of these insoluble products (e.g., Table II) indicated that they represented covalent peptide–protein conjugates coupled through the tyrosine of each (increased Met and/or Phe content, loss of tyrosine and nitrotyrosine, and no change in other amino acids). The pH 6.2 soluble products formed in the presence of peptides showed no evidence of significant peptide coupling. The pH 6.2 insoluble fractions were soluble at pH 3 and were further fractionated according to both size and peptide affinity (vide infra).

Fractionation of Peptide-Linked Products by Size. Fractionation on Sephadex G-50 indicated that all peptide-linked products contained a high proportion of covalent dimers and higher covalent oligomers (data not shown). Several protein peaks were obtained for each product. The most retarded corresponded to the elution volume of the native protein and was pooled separately; SDS gel electrophoresis demonstrated a molecular weight of 10 000–11 000 (data not shown). The faster moving components were collectively pooled and gave molecular weights of ~23 000, ~38 000, and ~52 000 on SDS gel electrophoresis, the relative proportions of each diminishing with increased molecular weight.

Fractionation of Peptide-Linked Products by Affinity Chromatography. The monomeric (M_r 10 000–11 000) and higher molecular weight components were separately affinity chromatographed at pH 3, in parallel with a control of native protein (Figure 7). Amino acid compositions of each peak were determined. Values for the monomers (Table II) did not differ significantly from those of the corresponding oligomers (data not shown). As indicated above, all fractions were peptide coupled, as evidenced by the extra Met and/or Phe content, with coupling occurring through Tyr as evidenced by

the loss of Tyr and NO_2-Tyr and the lack of alteration of other amino acids.[4] Different degrees of coupling were represented. The amino acid compositions of all Met-Phe-Tyr-NH_2 adducts indicated ~1 mol of coupled peptide/mol while those of Phe-Tyr-NH_2 and Tyr-Phe-NH_2 adducts gave values ranging from 2 to 3 mol of peptide/mol. All samples were polydisperse on HPLC. For example, the monomeric 1:1 adduct of Met-Phe-Tyr-NH_2 eluted as a broad multicomponent peak between 20 and 29 min under HPLC conditions compared to the 1.2-min peak width for the native protein (Materials and Methods). The different components are assumed to represent different modes of cross-linking.

Native neurophysin displays unusual heterogeneous behavior on affinity chromatography at pH 3, the largest fraction being barely retarded (Figure 7). By contrast, a large fraction of all the peptide-coupled components did not elute until the pH was lowered to 2.1 (Figure 7), indicating that a significant share had affinities equal to or greater than native protein. To determine whether the unretarded protein fractions contained nonbinding components, several of these from the initial affinity chromatography separation were resubjected to affinity chromatography at an initial pH of 3.2. The monomeric nonretarded adduct with Met-Phe-Tyr-NH_2 from the first chromatogram behaved essentially indistinguishably from native protein and from the initially retarded monomer from Phe-Tyr-NH_2 coupling (data not shown). Thus, the affinity chromatography behavior of the monomeric adduct of Met-Phe-Tyr-NH_2 at low pH does not indicate a significant content of subspecies with intrinsically lower binding affinity than native protein. this was also indicated by HPLC analysis (data not shown) which indicated that this product is a mixture of components that are largely all represented in each fraction of the affinity profile.[4]

In contrast to the monomeric adduct of Met-Phe-Tyr-NH_2, affinity rechromatography and HPLC demonstrated that the nonretarded monomer and polymer components of the Phe-Tyr-NH_2 adduct were significantly enriched in unique products of intrinsically lower binding affinity than the native protein (data not shown). However, the small quantity of this material and its degree of coupling (two peptides per chain) did not permit useful structural analysis.

Discussion

These results support the concept that neurophysin Tyr-49 is proximal to bound peptide and plays a role in peptide binding.[1] Thus, they demonstrate that modification of neurophysin by TNM can lead to products, selectively modified at Tyr-49, in which the basic elements of conformation and self-association are preserved, but in which the ability to bind peptides is lost. This is the first demonstration of such an effect. The loss of binding associated with affinity labeling of Tyr-49 is accompanied by loss of protein self-association (Abercrombie et al., 1984). In studies of Tyr-49 photooxidation (Fukuda et al., 1976a,b), the magnitude of the decrease in binding affinity was not calculated, and effects on conformation were not monitored. The persistence of self-association in the nonbinding TNM products is particularly significant. In native neurophysin, the Tyr-49 ring is proximal to the subunit interface, albeit not a direct participant in dimerization (Peyton et al., 1986). Thus, a selective negative effect of Tyr-49 modification on binding, as opposed to self-association, argues for a very local change that does not transcend the boundaries of the binding site. It is additionally relevant that the loss of binding cannot reflect modification of residues other than Tyr-49 that would be undemonstrable by amino acid analysis (e.g., deamidation). All mononitrated and cross-linked products of TNM treatment in the absence of peptide, which should be similarly vulnerable to such reactions, are functional.

A role for Tyr-49 in binding is in keeping with its evolutionary conservation [e.g., see Breslow (1979)] and apparent proximity to the peptide site as judged by both NMR and photoaffinity labeling (Peyton & Breslow, 1985; Abercrombie et al., 1982). What is remarkable are the variety of modifications of the Tyr-49 ring that can be achieved without significantly weakening peptide binding. Thus, for the most part, neither diiodination nor cross-linking of the ring leads to a large diminution in binding affinity; in fact, several cross-linked products show significantly increased binding affinity. These nondestructive modifications can be added to the list of prior ring modifications (see the introduction) that were without significant functional effects. The ability to so modify the ring without functional consequences, particularly segments at and adjacent to the hydroxyl, is consistent with the demonstration that the ring is largely exposed to solvent in the bound state (Griffin et al., 1973). It is also consistent with current estimates of distances between the ring and segments of bound peptide. These range from 5–10 Å to >13 Å for the distances to the side chains of peptide residues 1–3 (Sur et al., 1979; Lord & Breslow, 1980; Peyton et al., 1987). The question therefore arises as to the nature of the role played by Tyr-49 in binding.

We suggest that one role for Tyr-49, particularly the ring, is to maintain the apolar nature of the peptide binding site [e.g., see Breslow (1979)] by preventing access of solvent. Within limits of size, modifications that do not decrease the apolar nature of the ring should be compatible with peptide binding and would also, as observed here for several cross-linked products, increase binding affinity to the extent that shielding of the binding site from solvent was increased. In this context, loss of binding affinity by TNM treatment would be attributed to further modification of the nitrated ring to a more polar structure that permitted solvent access to the binding site. Alternatively, loss of binding affinity might reflect a role of the β-CH_2 or backbone region of Tyr-49 in interaction with peptide or other segments of the binding site, the nonbinding products of TNM treatment arising from modification of segments of Tyr-49 outside the ring. More specific identification of the side products of TNM treatment in model systems should allow distinction among these possibilities. It is relevant, however, that the nonbinding products we observe are spectrally similar above 300 nm to side products of tyrosine nitration by TNM observed by Boyd and Smith (1970) that are not cross-linked and which contain modifications of an unknown nature in part involving the α-amino group.

Oxidative attachment of Met-Phe-Tyr-NH_2 to Tyr-49 via TNM occurred in a 1:1 ratio. However, no significant nonbinding monomer was generated despite the apparent heterogeneity of modes of attachment. These results differ from the effects of labeling Tyr-49 with Met-Tyr-azido-Phe-NH_2, although both peptides are joined to Tyr-49 by peptide residue 3. A probable explanation of these different effects lies in differences in the mode of attachment to Tyr-49. Attachment

[4] The slight NO_2-Tyr content of some fractions is assigned to contaminating mononitrated protein as judged by HPLC analysis of Met-Phe-Tyr-NH_2 adducts. Retarded and nonretarded monomeric fractions from the affinity chromatogram had broad bands (Results) that were indistinguishable in profile except for a small additional peak in the retarded fraction with a mobility identical with that of mononitrated neurophysin II.

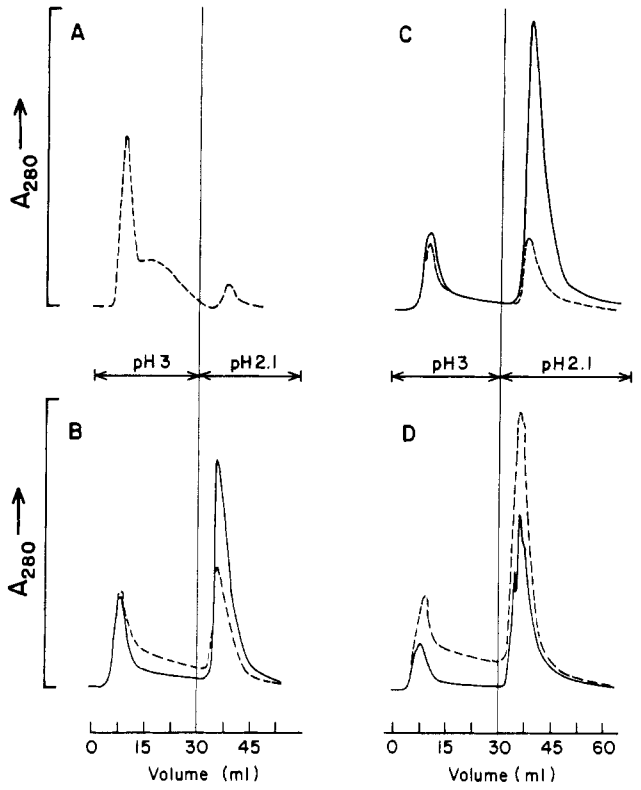

FIGURE 7: Affinity chromatography profiles of native neurophysin and peptide-coupled adducts at an initial elution pH of 3; the pH is lowered to 2.1 after 30-mL volume. (A) Native neurophysin II isolated as binding material from affinity chromatography at pH 6.2; (B) adducts of Met-Phe-Tyr-NH_2; (C) adducts of Phe-Tyr-NH_2; (D) adducts of Tyr-Phe-NH_2. In the chromatograms of the peptide-coupled fractions, monomer fractions (- - -) and fractions containing dimers and oligomers (—) are shown separately.

of Met-Phe-Tyr-NH_2 can be expected to be selective for the Tyr hydroxyl or positions ortho to the hydroxyl (Figure 5). Photoattachment of Met-Tyr-azido-Phe-NH_2 can, in principle, occur at any position. Accordingly, we assume that the latter did not occur at the positions available to Met-Phe-Tyr-NH_2. Given the unusual chemical reactivity of the adduct of Met-Tyr-azido-Phe-NH_2, which can rearrange to regenerate Tyr-49 (Abercrombie et al., 1982), a likely point of attachment is the ipso ring carbon; ipso-substituted rings have a tendency to regain aromaticity by rearrangement. In either event, the lack of effect of cross-linking Met-Phe-Tyr-NH_2 to the ortho ring protons of Tyr-49 is consistent with the relatively long distance between the side chain of peptide residue 3 and the ortho protons suggested by NMR (Lord & Breslow, 1980; Peyton et al., 1987). We suggest that the cross-linking and NMR studies are best reconciled with photoaffinity labeling studies by a model that places the side chain of peptide residue 3 closest to the Tyr-49 backbone or β-CH_2 segments rather than to the ring.

In contrast with the monomeric 1:1 adduct of Met-Phe-Tyr-NH_2, we find evidence of protein with reduced binding affinity among the 2:1 monomeric and oligomeric adducts of Phe-Tyr-NH_2. A nonexclusive explanation of these results is that substitution of both Tyr-49 ring positions ortho to the hydroxyl with very bulky groups sterically impedes binding of peptide residue 1. It is also possible that only one position on Tyr-49 is occupied by peptide, with the second peptide attached to the first and interacting with the binding site.

The present studies emphasize the accepted, but occasionally neglected, concept that a functional residue can often be modified, or substituted, with few functional consequences and that the significance of the effects of modification can best be appreciated by using different modifications or substitutions. Additionally, given the possibility of backbone interactions at least raised here, the point should probably also be made that side-chain modification alone is inadequate to ascertain the importance to function of a given residue. Further studies of Tyr-49 are needed to determine whether its functional role reflects principally the ring or segments outside of the ring.

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Registry No. Tyr, 60-18-4; Met-Phe-Tyr-NH_2, 106231-12-3; Phe-Tyr-NH_2, 38678-74-9; Tyr-Phe-NH_2, 38678-75-0.

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Allosteric Interactions in Sipunculid and Brachiopod Hemerythrins†

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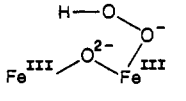
ABSTRACT: Chemical and spectroscopic consequences of allosteric interactions for ligand binding to sipunculid (*Phascolopsis gouldii*) and brachiopod (*Lingula reevii*) hemerythrins (Hrs) have been investigated. Possible allosteric effectors for homotropic effects in sipunculid Hrs have been examined, but only reduction in ligand affinity is observed without cooperativity. In contrast to sipunculid Hr, *L. reevii* Hr binds O_2 cooperatively in the pH range 7–8 and exhibits a Bohr effect. Spectroscopic comparisons of the sipunculid and brachiopod Hrs show no significant differences in the active site structures; therefore, modulation of oxygen affinity is attributable to effects linking the site to quaternary structural changes in the octamer. Oxygen equilibria can be fit with a conformational model incorporating a minimum of three states, tensed (T), relaxed (R), and an R–T hybrid. Resonance Raman spectra of *L. reevii* oxyHr show a shift in the peroxo stretching frequency when the pH is lowered from pH 7.7 (predominantly R oxyHr) to pH 6.3 (a mixture of R, T, and R–T hybrid), but *P. gouldii* Hr does not have a frequency shift under the same conditions. In contrast to hemoglobins, ligand binding to the deoxy and met forms is noncooperative for brachiopod (and sipunculid) Hrs. It is thus suggested that conformational changes in the protein are linked to the oxidation state change that accompanies oxygenation of the coupled binuclear iron site (deoxy [$Fe^{II}Fe^{II}$] → oxy [$Fe^{III}Fe^{III}$]). The total allosteric energy expended in oxygenation is about 1.4 kcal/mol, and such a shift is possible in the relaxed–tense conversion with relatively limited constraints of the iron coordination environment via the protein quaternary structure. The mechanism of cooperativity in the binuclear copper oxygen carrier hemocyanin is discussed in light of these results.

Hemerythrins are non heme iron proteins known to occur in four marine phyla, including *Sipunculida* and *Brachiopoda* (Klippenstein, 1980). As a reversible oxygen carrier, hemerythrin has been implicated in the transport and storage of oxygen in the sipunculids (Mangum & Kondon, 1975), but the precise relationship between its functional characteristics and physiological role has not been defined. This situation derives, in part, from the observation that the oxygen equilibrium curves of cellular hemerythrin are significantly different from those of purified extracts (Mangum & Kondon, 1975; Wells, 1982), with the $p_{1/2}$ values being higher for the intact coelomic cells. In addition, sipunculid cellular hemerythrin has been reported to have cooperativity in oxygen equilibrium that is absent in purified protein (Mangum & Kondon, 1975). These observations suggest the presence of allosteric effects in the ligand binding properties of hemerythrin, a topic that has not been addressed in significant detail. The study reported here involves an investigation of allosteric phenomena in purified octameric hemerythrins isolated from two sipunculids and a brachiopod.

The physical, structural, and spectroscopic properties of coelomic hemerythrins (Hrs) have been reviewed (Kurtz et al., 1977; Klippenstein, 1980; Loehr & Loehr, 1979; Klotz & Kurtz, 1984). A relatively recent development is the agreement by two X-ray crystallographic groups on the ligand environment in the binuclear iron active site. Presently, the oxidized site with bound azide (metazidoHr, [$Fe^{III}Fe^{III}N_3^-$]) is known to have the same general structure in the hemerythrins of two distinct species, *Phascolopsis gouldii* myoHr and *Themiste dyscritum* coelomic Hr (Stenkamp et al., 1984; Hendrickson et al., 1975). From single-crystal polarized spectra (Gay & Solomon, 1978), it was determined that the end-on binding mode found crystallographically for azide is retained in the oxy form, where dioxygen is bound as peroxide:



Extended X-ray absorbance fine structure (EXAFS) (Hendrickson et al., 1982) and low-resolution X-ray crystallographic studies (Stenkamp et al., 1985) have confirmed that the μ-oxo

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