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Conformational Flexibility of Neurophysin As Investigated by Local Motions of Fluorophores. Relationships with Neurohypophyseal Hormone Binding†

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ABSTRACT: Flexibility of various structural domains of neurophysin and neurophysin–neurohypophyseal hormone complexes has been investigated through the fast rotational motion of fluorophores in highly viscous medium. Despite seven intrachain disulfide links, it is shown that some domains of neurophysin remain highly flexible. Dimerization of neurophysin does not affect the structural integrity of the individual subunits, each subdomain being conformationally equivalent within each protomer of the unliganded dimer. The absence of heterogeneous fluorescence anisotropy precludes the existence of a dimer tautomerization equilibrium. Binding of the hormonal ligands to neurophysin dimer promotes a large conformational change over the whole protein structure as assessed by differential alterations of the flexibility–rigidity and intrasegmental interaction properties of domains that do not participate directly to the dimerization/binding areas. The order of free-energy coupling between ligand binding and protein subunit association has been evaluated. Data are consistent with a model in which the first mole of bound ligand stabilizes the dimer by increasing the intersubunit contacts while the second mole of ligand induces most of the described conformational change. Accordingly, the positive cooperativity between the two dimeric binding sites is linked mainly to the binding of the second ligand. The induced structural change is perceived differently by each subunit as assessed by opposite local motions of Tyr^{49} in each liganded protomer and leads to the formation of a dimeric complex with a global pseudospherical symmetry although containing domains of local asymmetry.

The posterior pituitary contains a class of highly disulfide-linked proteins, neurophysins, associated with both biosynthesis and transport of the neurohypophyseal hormones, ocytocin and vasopressin, along the hypothalamo neurohypophyseal tract [for recent reviews, see Cohen et al. (1983), Chaiken et al. (1983), Richter (1983), Breslow (1984), and Pickering & Swann (1984)]. Within the neurosecretory granule two distinct neurophysins are found in noncovalent association with a different hormone although each neurophysin is able to bind vasopressin or ocytocin with similar affinities in vitro.

Studies of the polymerization process of neurophysins demonstrated that these proteins self-associate in a form of

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a dimer in aqueous solutions (Nicolas et al., 1976, 1980; Pearlmutter, 1979). Both the insensitivity to pH and temperature of the dimerization equilibrium and kinetic measurements suggested that protomer association areas are mainly hydrophobic in nature (Pearlmutter, 1979; Nicolas et al., 1980). Hydrodynamic parameters of the monomer and the dimer favored an entropy-driven side-by-side association model allowing maximum contact between subunits and stabilization of the dimer (prolate ellipsoid of axial ratio a/b = 3.6) through numerous weak interactive forces (Rholam & Nicolas, 1981). Dimer formation seems to be achieved without profound conformational rearrangement in the monomer structure as assessed by spectroscopical evidence (Nicolas et al., 1978a, 1979; Sur et al., 1979).

Noncovalent complexes formed between neurophysins and hormones, or peptide analogues, have been submited to spectroscopical and hydrodynamic analysis. Facilitated dimerization is observed under conditions of preferential binding to the dimeric form of hormones and analogues (Nicolas et al., 1976, 1978a, 1980; Pearlmutter & McMains, 1977; Pearlmutter & Dalton, 1980) and positive binding cooperativity has been detected between the two strong dimeric sites common to both hormones (Hope et al., 1975; Nicolas et al., 1978a,b; Pearlmutter & Dalton, 1980; Tellman & Winzor, 1980). Spectroscopic studies have provided some evidence that complex formation should produce local rearrangements in the neurophysin tertiary structure. In particular, UV absorption spectra arising from the single tyrosine side chain in position 49 indicate that this moiety is displaced from a hydrophobic environment to a more polar one upon ligand binding (Griffin et al., 1973; Wolff et al., 1975). CD spectroscopy additionally indicated slight perturbation arising from neurophysin disulfide links (Breslow & Weiss, 1972). Recent hydrodynamic studies demonstrated that ligand binding leads to the formation of compact pseudospherical dimeric complexes (Rholam et al., 1982). Such a ligand-induced conformational change was postulated to be linked with the intradimeric cooperativity properties of the dimer. The real extent to which the tertiary structure of such a small, highly constrained, protein is flexible and is able then to accommodate large conformational perturbations is unknown. The above findings indicate that neurophysin tertiary structure has indeed some flexibility and undergoes more profound and extented changes upon binding than those detected through the limited Tyr^{49} and disulfide bridge spectroscopical probes.

In order to gain relevant and general information on the flexibility–rigidity properties of that particular class of proteins and to detect the structural changes induced by polymerization and/or binding, changes in local environmental flexibility of various subdomains of neurophysins and neurophysin–hormone complexes have been investigated through the modifications in the fast rotational motions of fluorophore residues in highly viscous medium following the new approach developed by Weber et al. (1984).

Materials and Methods

Highly purified neurophysins I and II were prepared by isoelectric focusing (Camier et al., 1973). Mononitrated Tyr^{49} neurophysins were obtained by reaction with tetranitromethane and purified by electrophoresis as previously reported (Wolff et al., 1975). The dansyl (Dns) fluorescent group was covalently attached to the protein through residues Ala^1 and Lys^{18} (or Lys^{59}) as previously reported (Rholam & Nicolas, 1981).

The tripeptides Cys(*S*-Me)-Tyr-Ile-NH_2 and Cys(*S*-Me)-Phe-Ile-NH_2 were from Bachem. Their spectroscopic purity was assessed by absorption and fluorescence measurements. Spectral-quality glycerol was from Aldrich.

Fluorescence polarization measurements were done with the apparatus described by Jameson et al. (1976). The values were corrected for solvent background if its contribution to the total intensity exceeded 0.5%. Fluorescence lifetimes were determined by the cross-correlation phase method (Spencer & Weber, 1969) with updated electronics from SLM (SLM Instruments, Urbana, IL). Corning 0-53 filters were used on tyrosyl emission, and the exciting light was isolated with Beckman monochromator set at 280 nm and a 7-54 Corning filter. For dansyl fluorescence, the monochromator was set at 350 nm, and Corning 3-73 filters were used for emission.

Temperature was regulated by a methanol circulating bath, and optical modules were purged with dry nitrogen to prevent frosting. Viscosity values for glycerol–water mixtures were from Miner & Dalton (1953).

Native and dansylated neurophysins were dissolved in 80% glycerol–20% phosphate buffer (0.05 M, pH 7.0). The polarization and lifetime of the solutions were determined in the interval −40 to 20 °C. The data were analyzed by employing the relation established in a previous paper (Weber et al., 1984):

$$Y = \ln [A(0)/A(T) - 1] - \ln [RT\langle\tau\rangle/V] = -\ln \eta(0) + b(T - T_0)$$

where $A(0)$ is the limiting anisotropy, $A(T)$ is the value of the anisotropy at the Kelvin temperature T, R is the gas constant, $\langle\tau\rangle$ is the mean lifetime of the excited state, V is the effective volume of the fluorophore, $\eta(0)$ is the viscosity at the temperature T_0 and b is the thermal coefficient of the "local" viscosity that characterizes the resistance that the environment offers to the rotation of fluorophores.

In the general case (Rholam et al., 1984; Scarlata et al., 1984), the plot of Y against the temperature $t = (T - T_0)$ yields two slopes corresponding to two regions in which the rotational motions of the fluorescent residue are limited either by the external solvent [b(S)] or by the peptide environment [b(U)], respectively.

Results

Local Motions in Neurophysins. The Y plot of native neurophysin against the temperature t is presented in Figure 1. This plot is characterized by two linear parts corresponding to two different regimes (Rholam et al., 1984; Scarlata et al., 1984): one at low temperature whose slope is equal to the thermal coefficient of the solvent [b(S) = 7%] and the other one at high temperature whose thermal coefficient is much smaller [b(U) = 3.2%]. The transition from one region to the other occurs at a critical temperature (t_c = −12 °C) where the motions of the tyrosyl groups become restricted by the protein environment.

As it is shown in Figure 2, a similar pattern is obtained for dansylated neurophysin except that two high-temperature slopes are observed: $\langle b(U)\rangle_1$ = 4.3% and $\langle b(U)\rangle_2$ = 5.9%. This result was expected since each subunit has two distinct sites for dansyl labeling, Ala^1 and Lys^{18} (or Lys^{59}) (Rholam & Nicolas, 1981). On the basis of the values of both the apparent thermal coefficients, $\langle b(U)\rangle$, and the relative contributions of each fluorophore to total fluorescence emission, f(i) (Rholam & Nicolas, 1981), the values of b_i(U) have been calculated by the relation previously reported (Scarlata et al., 1984).

The same data (Table IA) have been obtained for both native and dansylated neurophysin, under conditions in which either the monomer or the dimer is the prevalent species (Nicolas et al., 1980). This finding agrees with the results

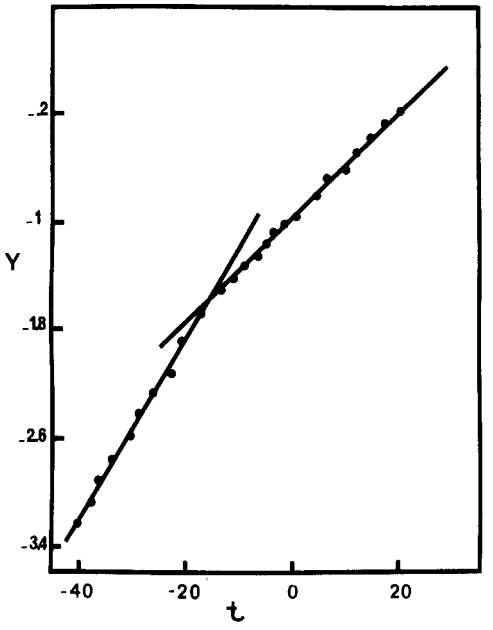

FIGURE 1: Plot of Y vs. temperature t of the native neurophysin dissolved in 80% glycerol–20% 0.1 M acetate buffer, pH 6.2.

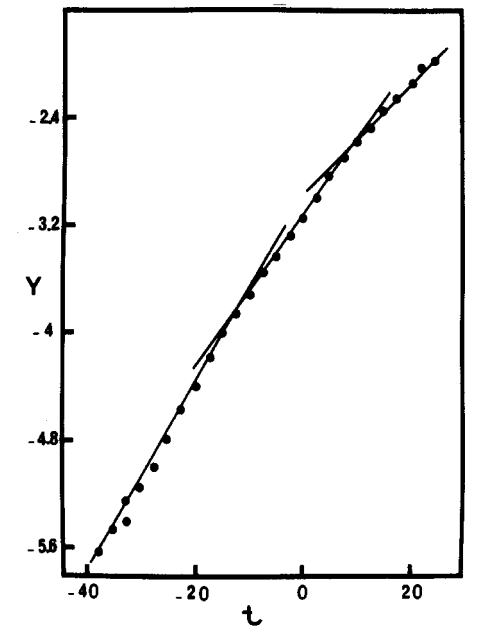

FIGURE 2: Plot of Y vs. temperature t of the dansylated neurophysin under the same conditions as given in Figure 1.

obtained by other methods (Pearlmutter & Dalton, 1980; Rholam & Nicolas, 1981) and indicates that tyrosyl and dansyl immediate environments are unperturbed upon dimerization.

Ligand Effects on Local Motions. Since neurophysins contain a strong site per monomer that binds hormones or peptide analogues (Nicolas et al., 1978a), two N-terminal tripeptide analogues of ocytocin have been used to analyze the rotational motions of the tyrosyl groups of neurophysins in the following dimeric complexes:[1] (A) NO_2-Tyr^{49}–Tyr^2; (B) Tyr^{49}–Phe^2; (C) Tyr^{49}–Tyr^2. As the fluorescence of the tyrosine in neurophysin can be completely quenched by nitration (NO_2-Tyr^{49}), the motions of Tyr^2 of the bound substrate can be examined by following the fluorescence depolarization of

[1] Emission from any nonbound ligand or exposed tyrosyl groups was quenched by addition of a 100-fold molar excess of sodium citrate (final concentration 0.1 M).

Table I: Neurophysin Parameters Obtained in the Presence or Absence of Peptide Analogues

species	$100\langle b(U)\rangle$ (°C^{-1})	$100b(U)$ (°C^{-1})	t_c (°C)	amplitude (deg)[a]
(A) Neurophysins				
neurophysin		3.2	−12	13
neurophysin-Dns[b]	4.3	4.9 (i)	8	26
	5.9	2.2 (ii)	−12	13
(B) Neurophysin Complexes				
NO_2-Tyr^{49}–Tyr^2		5.5	10	28
Tyr^{49}–Phe^2	3.4		5	23
	5.35		−26	7
Tyr^{49}–Tyr^2	4.5		8	26
	5.4		−26	7
neurophysin-Dns–Tyr^2		4.1	−3	18

[a] The amplitude was calculated from the relation determined in a previous paper (Rholam et al., 1984): $\langle\cos\Phi\rangle^2 = [1 + 2A(t_c)/A(0)]/3$. [b] The parameter values of each fluorophore, (i) Dns-Ala^1 and (ii) Dns-Lys^{18} or Dns-Lys^{59}, were determined from the relation (Scarlata et al., 1984) $\langle b(U)\rangle = \sum f(i)b_i(U)$.

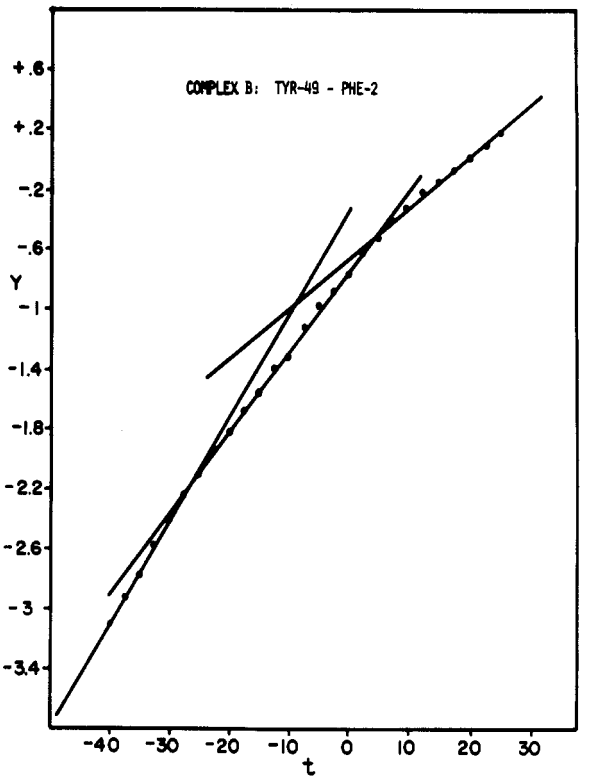

FIGURE 3: Y plot of the neurophysin–Cys(*S*-Me)-Phe-Ile-NH_2 complex. The experimental conditions are the same as those in Figure 1.

Cys(*S*-Me)-Tyr-Ile-NH_2 liganded to nitrated neurophysin dimer (complex A). On the other hand, the motions of Tyr^{49} in the neurophysin were analyzed through the fluorescence depolarization of the native protein complexed with the tripeptide Cys(*S*-Me)-Phe-Ile-NH_2 (complex B). Complex C allowed the observation of motions of tyrosine of both protein and substrate. These experiments were conducted in the presence of saturating amounts of tripeptides (ligand to protein molar ratio = 10).

One high-temperature slope is observed for complex A (Table IB) whereas two high-temperature slopes characterize complex B (Table IB and Figure 3). Hence, we expected to observe three high-temperature slopes for complex C corresponding to a linear combination of those obtained in complexes A and B. However, due to very close critical temperature values of Tyr^2 and one of the Tyr^{49} residues in the dimer

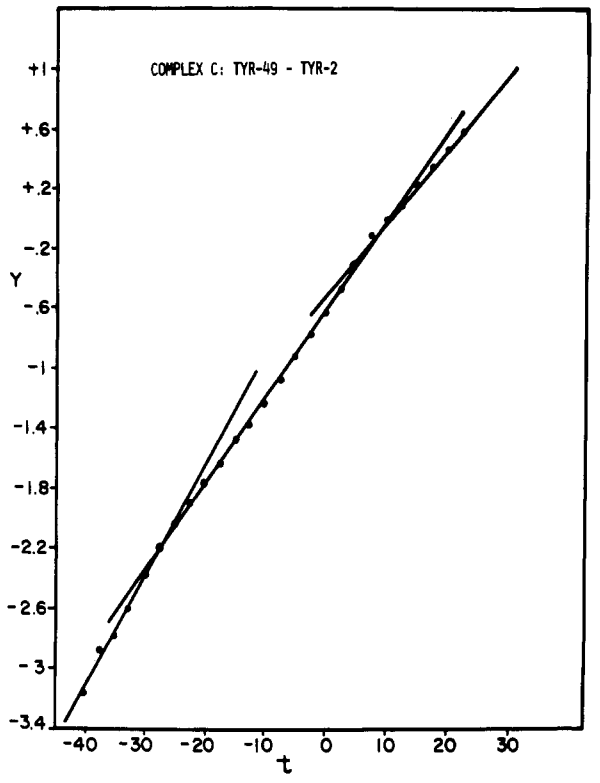

FIGURE 4: Y plot of the neurophysin–Cys(S-Me)-Tyr-Ile-NH_2 complex. The conditions used are the same as those in Figure 1.

(Scarlata et al., 1984), only two thermal coefficient values of $\langle b(U)\rangle$ have been determined (Table IB and Figure 4). These observations indicate that the Tyr[2] groups are located in two identical domains that correspond to the high-affinity dimeric sites. Moreover, the ligands act differently on the conformation of neurophysins so that the dimeric environments of Tyr[49] groups (one per monomer) become unequivalent.

In contrast, the analysis of local motions of the dansyl residues of neurophysin indicates that, upon ligand binding, only one value of the parameters, t_c and $b(U)$, can be determined (Table IB). Thus, upon ligand binding the dansyl groups move into environments that are not experimentally distinguishable. Also, the hormonal ligands produce the same effect in each protomer of the liganded dimer.

Order of Free-Energy Couplings. While the binding of peptide ligands to neurophysins is cooperative (Nicolas et al., 1978a,b) and promotes pseudospherical complexes (Rholam et al., 1982), the mechanism by which this intradimeric cooperativity occurs is still unknown. In view of the importance of the relation between this phenomenon and the above data, the order of free-energy couplings between ligand binding and protein subunit association has been analyzed by the following equation (Weber, 1970, 1972, 1984):

$$I = \langle \Delta G_{22} - \Delta G_{11} \rangle / \langle \Delta G_{21} - \Delta G_{11} \rangle$$

where ΔG_{11} designates the standard free energy of ligand binding to the monomer while ΔG_{21} and ΔG_{22} are respectively the standard free energies of association of the first and second molecule of ligand L to the dimer. If $I = 1$, the free-energy couplings are of intermediate order. If $I \gg 1$, they are practically of second order, and if $I \ll 1$, they are predominantly of first order.

From the values of standard free energies (Table II), a value equal to 3 is obtained for the parameter I. According to the above, the coupling effect in the neurophysin dimer is of second order. Therefore, the observed positive cooperativity is essentially produced by the binding of the second mole of ligand, which induces a maximum conformational rearrangement in the structure of neurophysin dimer (Rholam et al., 1982).

Table II: Free Energies of Ligand Binding and Dimerization Process of Neurophysin at T = 298 K

equilibrium	association constants (mol^{-1})[a]	free energies (kcal mol^{-1})
$M + L \rightleftharpoons ML$	$k_1 = 6.11 \times 10^4$	$\Delta G°_{11} = -6.53$
$D + L \rightleftharpoons DL$	$k_2 = 1.3 \times 10^5$	$\Delta G°_{21} = -6.98$
$DL + L \rightleftharpoons DL_2$	$k'_2 = 5.35 \times 10^5$	$\Delta G°_{22} = -7.82$

[a] The constant values were taken from Nicolas et al. (1978b).

Discussion

Conformational Flexibility of Neurophysins. According to the interpretation of the thermal coefficient of the viscosity and the critical temperature parameters (Rholam et al., 1984; Scarlata et al., 1984), obtained for some neurophysin fluorophores, the present study leads us to deduce conformational informations about the domains interactions of neurophysins in the absence or in the presence of hormonal ligands.

A low value of $b(U)$ was obtained for the Dns-Lys[18] (or Lys[59]) and Tyr[49] residues. This result was interpreted as reflecting strongly restricted local motions of fluorophores by the tertiary structure of their immediate peptide neighbor. Indeed, the obtention of small amplitude values indicates that the local environment around these fluorophores behaves like a flexible conformational domain. In addition, identical parameters values were found for these fluorophores, suggesting that their local environments are common or nearly equivalent. In contrast, much higher parameters values have been found for the other dansyl attached to Ala.[1] Thus, according to the above, the Dns-Ala[1] local motions undergo less interactions with its peptide environment, whose structure is highly rigid. As the local rotation of a fluorophore is determined by its immediate surrounding, comprising a domain of some 10 amino acid residues (Scarlata et al., 1984), data clearly indicate that the Ala[1]–Cys[10] segment is strongly motionnally restricted by intrasegmental interactions. By analogy with the data reported for vasopressin, pressinoic acid, and somatostatin (Rholam et al., 1984), the presence of some specific interactions in the 1–10 domain, results in damping the coupled motions of fluorophores and their peptide environment. In this respect, analysis according to Chou & Fasman showed a high α-helix-forming potential for the 1–9 segment of either neurophysin I or II (Nicolas, 1979).

Neurophysin dimerization involves hydrophobic interactions confined within the conserved common sequence 10–74 of the protein (Nicolas et al., 1980). Refined theoretical analysis suggest that dimerization areas are located within residues 21–42 and/or 65–80 of the protein sequence (unpublished data), excluding almost totally direct involvement of both the central core of the protein (Residues 45–60) and its N- and C-terminal regions. The fact that the local environments of Dns-Lys[18] (or Lys[59]), Dns-Ala[1], and Tyr[49] fluorophores were found independent of the protein concentration in a region where neurophysins dimerize reversibly brings additional evidence for the above proposal. Moreover, it supports the previous view (Rholam & Nicolas, 1981) of a side-by-side dimerization of neurophysin, which is achieved without major conformational change in the protomer structure. On the basis of the above, recent observations that loss of residues 1–8 decreases both ligand binding and neurophysin self-association (Sardana & Breslow, 1984) could be explained by assuming that, although the folded 1–10 sequence is not part of either the ligand binding sites or the dimerization areas, it plays a major role in conformational stabilization of these recognition

domains through specific intersegmental interactions.

Analysis of motional parameters of neurophysins recorded in the presence of saturating amounts of hormonal ligands or tripeptide analogues shows multiple distinct effects on the above-described domains of the protein. Through the parameter values of the Tyr^2 residues of tripeptide analogue, when bound to neurophysin dimer, it is shown that the liganded hormone binding domain is highly rigid. Moreover, each protomer binding domain is conformationally equivalent in the dimer as seen by the appearance of a single value of both parameters, $b(U)$ and t_c. Most interesting is the comparison of the parameter values of unliganded vs. liganded dimeric neurophysin subdomains comprising Dns-Ala^1, Dns-Lys^{18}, (or Lys^{59}), and Tyr^{49}. The highly rigid 1–10 segment becomes flexible when ligand is bound to the two high-affinity dimeric sites. On the other hand, the Dns-Lys^{18} (or Lys^{59}) local environment raises in rigidity while decreasing its interactions with the rotational motion of this fluorophore in the liganded state. Since both fluorophores move oppositely to a similar degree of local environmental flexibility upon binding, it could be assumed that they reach nearly equivalent environments in the liganded dimer. Moreover, the existence of a single value of $b(U)$ and t_c for both fluorophores demonstrates the equivalence of each protomer domains in the dimer. In contrast, while both Tyr^{49} domains are equivalent and highly flexible in the unliganded dimer, each Tyr^{49} fluorophores move oppositvely from the other, one local environment becoming very flexible and the other one rigid. Thus, contrary to the situation encountered with other domains, the liganded neurophysin dimer is seen as asymmetrical through the Tyr^{49} probe.

Previous hydrodynamic studies (Rholam et al., 1982) have demonstrated that binding of neurohypophyseal peptides to neurophysin dimer promotes the formation of a compact and pseudospherical complex as a consequence of a putative ligand-induced conformational change. Spectroscopical studies have provided evidence that complex formation induces local rearrangements within the neurophysin tertiary structure although the restricted spectroscopic windows used did not allow an oveall view of these changes. The present data highlights profound alterations in the entire neurophysin tertiary structure arising from ligand binding and bring new insight in the dimerization process. While regions around Ala^1, Tyr^{49}, and Lys^{18} (or Lys^{59}) were shown not to participate directly in the dimerization contacts, the local conformational of the 1–10 segment are of the utmost importance in conformational stabilization of area involved in promoter–promoter interactions and/or ligand recognition. Each of these sequentially distant domains undergoes profound alterations upon ligand binding, all of those but Tyr^{49} domain being equivalent in each promoter of the dimer. This last finding supports the hypothesis that local asymmetry is induced by ligand binding to the preexistant symmetrical dimer.

Relationships with the Intradimeric Cooperativity. The binding of hormonal ligands to the two high-affinity dimeric sites generated downward curvilinear Scatchard plots (Nicolas et al., 1978a,b; Tellman & Winzor, 1980) that were interpreted as reflecting positive intradimeric cooperativity between these sites. These data alone precluded any clear-cut choice between the preexistent isomerization model (Monod et al., 1965) and the ligand-induced cooperativity model (Koshland et al., 1966). The above-desribed ligand-induced conformational changes may provide a structural basis for the theoretical interpretation by which occurs the intradimeric cooperativity. Through the various spectroscopical probes distributed along the whole neurophysin structure, it is clear that each of their surrounding domains are conformationally equivalent between each promoter of the dimer in the unliganded state. Moreover, both this absence of heterogeneous anisotropy values (Table IA) and theoretical analysis (Weber, 1970, 1972, 1984) strictly exclude the existence of unliganded dimeric tautomers. This strongly favors a ligand-induced cooperativity scheme as working model.

We have thus further analyzed the intradimeric cooperativity phenomenon by evaluating the order of free-energy couplings between ligand binding and protein subunit association. According to the current theory (Weber, 1984), data are consistent with a model in which the first molecule of ligand stabilizes the neurophysin dimer by increasing protomer–protomer interactions through minor structural rearrangements of the protomer recognition area, while the second mole of ligand promotes a larger conformational change over the whole dimer structure. Therefore, the observed positive cooperativity is essentially generated by the binding of the second mole of ligand. In addition, each subunit perceives this induced structural change asymmetrically as assessed by different local motions of tyrosyl and dansylated residues in each promoter of the dimer. This could be interpreted as reflecting incomplete transmission of the change induced in the second subunit to the first upon binding of the second ligand due to loose protomer–protomer interactions.

Taken as a whole, these data provide a clear example of a small protein containing a high proportion of disulfide links and that still allows a considerable internal flexibility to some segments. Finally, with this methodology (Weber et al., 1984), a dynamic view of the tertiary structure of neurophysins can be envisioned by analyzing the local motions of fluorophores judiciously distributed along the peptide backbone of the protein.

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Partial Retro–Inverso Analogues of Somatostatin: Pairwise Modifications at Residues 7 and 8 and at Residues 8 and 9†

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ABSTRACT: Peptide bonds between residues 7 and 8 and residues 8 and 9, postulated internal cleavage sites of the peptide hormone somatostatin, were subjected to "pairwise" retro–inverso modification, where atoms of these peptide bonds were interchanged to give the analogues [*g*Phe^7-*m*-(*RS*)-Trp^8]somatostatin (I) and [*g*Trp^8-*m*-(*RS*)-Lys^9]somatostatin (II). Key fragments containing the modifications were synthesized by using [bis(trifluoroacetoxy)iodo]benzene for the generation of *gem*-diaminoalkyl-containing precursors from peptide amides. The versatility of solution synthetic methods was utilized to allow the incorporation of the modified segments. Protecting groups, removable selectively and under mild conditions, included *tert*-butyl-based groups for the side chains and the *tert*-butylmercapto group for the cysteine thiols. The excellent results obtained in the syntheses of analogues I and II, and previously of somatostatin on a larger scale [Moroder, L., Gemeiner, M., Goehring, W., Faeger, E., Thamm, P., & Wunsch, E. (1981) *Biopolymers 20*, 17–31], suggest the general feasibility of this route for the synthesis of centrally modified analogues. The purification of the products by Sephadex LH-20 chromatography afforded the separation of diastereomers of both analogues. The two isomers of I showed significant but different activities while those of analogue II were marginally active.

The disulfide-linked cyclic tetradecapeptide somatostatin is an important regulatory factor responsible for the inhibition of the release of growth hormone, insulin, glucagon, gastrin, and other peptide hormones. The in vivo half-life of somatostatin is only a few minutes due to rapid degradation by exo- and endopeptidases (Brazeao et al., 1974). In vitro experiments with brain homogenates suggested that the major internal cleavage site is between residues Trp^8 and Lys^9, in addition to the minor sites between residues Phe^6 and Phe^7 and residues Thr^{10} and Phe^{11} (Marks & Stern, 1975; Marks et al., 1976). These cleavages all occur within the central 6–11 region which has been established through structure activity studies as essential for biological function (Vale et al., 1978)

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