Synthesis of Peptide Spin-Labels That Bind to Neurophysin and Their Application to Distance Measurements within Neurophysin Complexes†

Susan T. Lord‡ and Esther Breslow*

ABSTRACT: The synthesis of two spin-labels capable of binding to the hormone-binding site(s) of neurophysin is described. The two spin-labels are 4-(glycyl-L-phenylalanylamido)-2,2,6,6-tetramethylpiperidinyl-1-oxy and *S*-[[[3-(2,2,5,5-tetramethylpyrrolidine-1-oxy)amino]carbonyl]methyl]-L-cysteinyl-L-tyrosine amide; synthesis of the former is achieved by a novel route to circumvent problems associated with nitroxide instability under standard conditions of peptide deblocking. NMR studies of the effects of binding these spin-labels on relaxation rates of individual proton resonances of neurophysin were used to calculate correlation times and distances between the bound nitroxides and the observed protons. The results indicate that residue 3 of peptides bound to the strong site of neurophysin is $\geq$ 14 Å from Tyr-49 and argue against a distance of <5 Å between the ortho ring protons of Tyr-49 and those of residue 2 of peptides bound to the strong site. Alternatively, the data suggest that the previously observed nuclear Overhauser effect between these protons reflects spin diffusion at the strong site and a contribution of uncertain magnitude from a second but very weak binding site; this second site is close to Tyr-49 and is detected by the increased relaxation rate of Tyr-49 ring protons when 4-(glycyl-L-phenylalanylamido)-2,2,6,6-tetramethylpiperidinyl-1-oxy is displaced from the strong site by competing diamagnetic peptide. Additionally, the data indicate that residue 3 of bound peptides at the strong site is distant from His-80 but ~12 Å from the amino terminus. The extended side chain of residue 1 of peptides at the strong site is calculated as $\leq$10 Å from Tyr-49.

Neurophysins are small proteins (M_r = 10 000) which interact in vivo with the posterior pituitary hormones oxytocin and vasopressin and in vitro also with small peptide analogues of the amino-terminal region of the hormones [for recent reviews see Walter (1975) and Breslow (1979)]. Each neurophysin polypeptide chain contains internally homologous segments comprised of residues 12–31 and 60–77 (Capra et al., 1972). Several investigations have provided evidence suggesting that, in addition to the principal hormone- or peptide-binding site on each chain, there is a second generally weaker binding site that is variably expressed (Camier et al., 1973; Hruby et al., 1975; Nicolas et al., 1976); this second site has been measured by equilibrium dialysis in the case of the hormones but has not been demonstrable by comparable studies in the case of the smaller peptides (Chaiken et al., 1975; Cohen et al., 1975; Lundt, 1977). The apparent potential for two binding sites and the presence of internal duplication has led to the suggestion that the duplicated regions may represent different binding sites (Camier et al., 1973); this concept is supported by preliminary affinity labeling studies which place the active-site carboxyl within one of the duplicated domains (Walter & Hoffman, 1973). Nonetheless, marked thermodynamic and spectroscopic differences between the sites (Breslow et al., 1973) suggest that other regions of the protein play a direct or indirect role in binding.

Each neurophysin that has been sequenced to date contains a single tyrosine (Tyr-49) in the middle of the central nonduplicated region of the polypeptide chain [e.g., Breslow (1979)]. Upon binding either the hormones or smaller peptides to the principal hormone-binding site, Tyr-49 is markedly perturbed (Furth & Hope, 1970; Breslow & Weis, 1972; Griffin et al., 1973). NMR[1] chemical shift and T_2 studies (Balaram et al., 1973) and UV absorption spectroscopy (Griffin et al., 1973), respectively, suggest that Tyr-49 is less restricted in the complex than in the unliganded protein and more exposed to solvent; however, nuclear Overhauser studies have suggested proximity in the complex between the ortho ring protons of Tyr-49 and the ring protons of the aromatic residue in position 2 of bound tripeptides (Balaram et al., 1973). Because only the strong hormone-binding site binds tripeptides with thermodynamically significant affinity (vide supra), the NOE data have suggested that Tyr-49 is adjacent to the strong hormone-binding site. However, no precise distance calculations are possible from the NOE data. Moreover, more recent insights into the nuclear Overhauser effect in macromolecular systems indicate that spin diffusion can spread the NOE over relatively long distances (Bothner-By & Johner, 1977).

In order to probe the distance between Tyr-49 and bound peptides more precisely, we have synthesized two peptide nitroxide spin-labels capable of binding to the principal hormone-binding site of neurophysin and studied the effects of their binding on the relaxation rates of the ring protons of Tyr-49 [see Krugh (1976) for a review of this technique]. The two spin-labels we prepared are A, 4-(glycyl-L-phenylalanylamido)-2,2,6,6-tetramethylpiperidinyl-1-oxy and B, *S*-[[[3-(2,2,5,5-tetramethylpyrrolidine-1-oxy)amino]-carbonyl]methyl]-L-cysteinyl-L-tyrosine amide. Electron spin resonance and other studies indicating that these spin-labels bind to the principal hormone-binding site of neurophysin and interact with neurophysin with apparent 1:1 stoichiometry have

† From the Department of Biochemistry, Cornell University Medical College, New York, New York 10021. *Received March 14, 1980.* Supported by Grant GM 17528 from the National Institutes of Health.

‡ Present address: National Cancer Institute, National Institutes of Health, Bethesda, MD 20205.

[1] Abbreviations used: NMR, nuclear magnetic resonance, NOE, nuclear Overhauser effect; TLC, thin-layer chromatography, ESR, electron spin resonance; NH_2-Tempo, 4-amino-2,2,6,6-tetramethylpiperidinyl-1-oxy; S-Tempo, 4-succinylamido-2,2,6,6-tetramethylpiperidinyl-1-oxy; spin-label A, 4-(glycyl-L-phenylalanylamido)-2,2,6,6-tetramethylpiperidinyl-1-oxy; spin-label B, *S*-[[[3-(2,2,5,5-tetramethylpyrrolidinyl-1-oxy)amino]carbonyl]methyl]-L-cysteinyl-L-tyrosine amide; S-CH_3-CFI, *S*-methyl-L-cysteinyl-L-phenylalanyl-L-isoleucine amide; EDTA, ethylenediaminetetraacetic acid; Cbz, carbobenzoxy.

0006-2960/80/0419-5593$01.00/0

been reported (Lundt & Breslow, 1976). Additionally, we have presented in preliminary form the results of our T_1 studies with these spin-labels which suggest the presence of a second and very weak peptide binding site adjacent to Tyr-49 (Lord & Breslow, 1978) and have reported effects of spin-label A on the line width of the neurophysin amino-terminal alanine methyl resonances (Lord & Breslow, 1979). The present report has two principal purposes. First, we wish to present in detail the synthetic route used to prepare the spin-labels. In trying to synthesize A we found that most techniques of peptide synthesis are incompatible with the integrity of the nitroxide spin-label and many techniques of spin-label synthesis are incompatible with the integrity of the peptide α-amino [which in the case of ligands to neurophysin is an essential group [e.g., Breslow (1979)]]. Second, we wish to elaborate on the implications of the data for the relative orientation of spin-labels on the protein surface and for the distance between Tyr-49 and the principal hormone- or peptide-binding site. Additionally, we will present further spin-label evidence (T_2 data) supporting the presence of a second binding site for the peptides.

Materials and Methods

NMR Studies. Bovine neurophysin I was purified as described previously (Breslow et al., 1971), separated from residual low molecular weight contaminants on Sephadex G-25 in 0.1 N acetic acid, and lyophilized. For NMR studies, the protein was subjected to repeated lyophilization from D_2O and then dissolved in D_2O at a concentration of 25–50 mg/mL in the presence of ~0.1 M NaCl and 10^{-3} M EDTA; the pH of all studies here was 6.2 (pH meter reading in D_2O).

The peptides *S*-methyl-L-cysteinyl-L-phenylalanyl-L-isoleucine amide and L-seryl-L-tyrosine amide were those previously described (Breslow et al., 1973) and were deuterated prior to NMR studies by lyophilization from D_2O. NH_2-Tempo (4-amino-2,2,6,6-tetramethylpiperidinyl-1-oxy) was purchased from Aldrich Chemical Co. Succinylated NH_2-Tempo (S-Tempo) and spin-labels A and B were synthesized as described below. Nitroxide concentrations were determined by using a Varian Model E-9 spectrometer with NH_2-Tempo solutions as standards as previously described (Lundt & Breslow, 1976); in the presence of protein, spin-label concentrations were determined at low pH where binding is negligible.

NMR studies were performed in 5-mm tubes on the 220-MHz Varian spectrometer at Rockefeller University using an external standard of 3-(trimethylsilyl)propionic acid in D_2O. Apparent T_2 values (T_2') were obtained from the widths of the peaks at half-height as described in the text. T_1 values were determined by the inversion–recovery method (Vold et al., 1968), fitting the data with computer programs supplied by Nicolet.

Synthesis of S-Tempo (4-Succinyl-2,2,6,6-tetramethylpiperidinyl-1-oxy). To a stirring mixture of 43 mg of 4-amino-2,2,6,6-tetramethylpiperidinyl-1-oxy (NH_2-Tempo) and 7 mL of water (pH 8.8) was added 40 mg of succinic anhydride over a 30-min period. After stirring for 1 h, the solution was lowered to pH 3.3 with glacial acetic acid, and the products were separated by chromatography on Sephadex G-10 in 0.1 M acetic acid. Analysis of the fractions by thin-layer chromatography (see below) indicated the appearance of a new spot moving with an R_f of almost 1. The product fractions containing no detectable starting spin-label were pooled and lyophilized to give a glassy orange product.

Synthesis of Spin-Label A [4-(Glycyl-L-phenylalanylamido)-2,2,6,6-tetramethylpiperidinyl-1-oxy]. (*a*) *4-(Carbobenzoxyglycyl-L-phenylalanylamido)-2,2,6,6-tetramethylpiperidine Hydrochloride.* To a solution of 1.0 g of dried Cbz-Gly-L-Phe (Vega Chemicals) and 0.86 g of 1-hydroxybenzotriazole (Aldrich) in 8 mL of cold, dried tetrahydrofuran was added 0.63 g of *N,N'*-dicyclohexylcarbodiimide in 3 mL of cold, dried tetrahydrofuran. After stirring on ice (4 h), the reaction mixture was warmed to room temperature and the urea precipitate removed by filtration. A solution of 0.86 g of 4-amino-2,2,6,6-tetramethylpiperidine (Aldrich) in tetrahydrofuran was added dropwise to the stirring filtrate. During piperidine addition, the solution was bubbled with nitrogen to prevent formation of the piperidine carbonate salt. The reaction was stirred at room temperature for 2 h and then refrigerated overnight. This coupling procedure is a modification of that described by Konig & Geiger (1970). The solvent was removed in vacuo and the white solid dissolved in a mixture of ethyl acetate and 0.1 N sodium bicarbonate. The bicarbonate layer was discarded and the ethyl acetate phase washed with water. The peptide was extracted from ethyl acetate with an equal volume of 0.1 N HCl. After refrigeration, white crystals were collected by filtration. This hydrochloride salt of 4-(carbobenzoxyglycyl-L-phenylalanylamido)-2,2,6,6-tetramethylpiperidine was recrystallized from hot methanol: yield 565 mg, 40%, mp 284–285 °C dec. The recrystallized carbobenzoxypeptide migrated as a single spot, R_f = 0.45, on thin-layer chromatography (see below).

(*b*) *4-(Glycyl-L-phenylalanylamido)-2,2,6,6-tetramethylpiperidine.* The carbobenzoxy protecting group was removed from 565 mg of hydrochloride salt product by catalytic hydrogenation over 10% palladium on charcoal in acidic ethanol. The reaction was stirred at room temperature (8 h) until CO_2 evolution stopped and a single, ninhydrin-reactive spot was seen on TLC. The solvent was removed in vacuo and the product mixture dissolved in water. This solution was evaporated in vacuo and the process repeated several times to remove the toluene produced by decarbobenzoxylation. The product was lyophilized from water: yield 335 mg. The product moved on TLC as a single, ninhydrin-positive spot, R_f = 0.1.

(*c*) *4-(Glycyl-L-phenylalanylamido)-2,2,6,6-tetramethylpiperidinyl-1-oxy.* 4-(Glycyl-L-phenylalanylamido)-2,2,6,6-tetramethylpiperidine (97 mg) was dissolved in 18 mL of water and the pH adjusted to 8.0 with 6 N NaOH. Citraconic anhydride (90 μL) was added over a period of 30 min while maintaining the pH at 8.0 by addition of 6 N NaOH. To this solution was added 15 mg of $Na_2WO_4 \cdot 2H_2O$, 15 mg $Na_2EDTA \cdot 2H_2O$, and 3 mL of 30% H_2O_2. The oxidation of the piperidine to the nitroxide was based on the method of Weiner (1969). The pH was adjusted to 9.5 and the reaction left at room temperature in the dark for 3 days. The oxidation was terminated and the citraconyl group removed by adjusting the solution to pH 3 with glacial acetic acid. After 18 h, the reaction solution was concentrated in vacuo and the product separated by chromatography on Sephadex G-10 eluted with 0.1 M acetic acid. The product fractions were pooled and lyophilized: yield 65 mg, 76% of the piperidine starting material. Two ninhydrin-positive spots were seen on TLC: R_f = 0.6, the major product, and R_f = 0.5, a minor product. The minor component was identified as the hydroxylamine reduction product of the nitroxide (major component) by showing that the major component was converted to the minor component by ascorbate. Additionally, it was demonstrated that the hydroxylamine was largely formed during thin-layer chromatography (Lundt, 1977). Amino acid analysis indicated that the product contained glycine and phenylalanine in a ratio of 1:1. Comparison of the ESR signal of the final product with

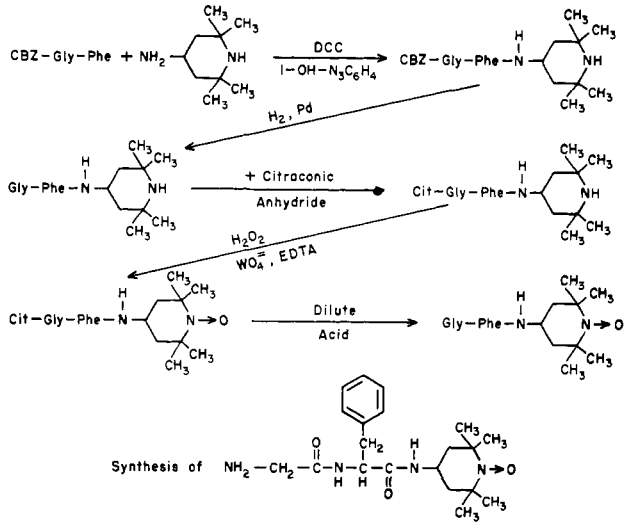
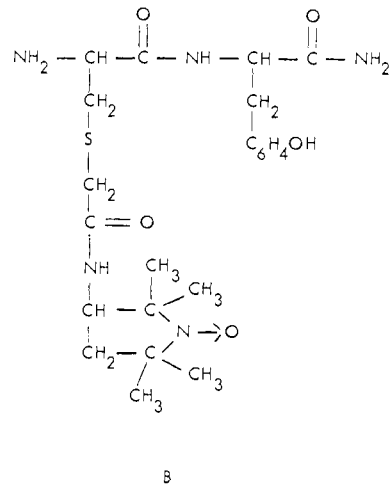


FIGURE 1: Synthesis of spin-label A.



B

FIGURE 2: Structure of spin-label B.

its amino acid content indicated that the hydroxylamine form represented <10% of the peptide.

Synthesis of Spin-Label B [*S*-[[[*3*-(*2,2,5,5-Tetramethylpyrrolidinyl-1-oxy*)*amino*]*carbonyl*]*methyl*]-L-*cysteinyl*-L-*tyrosine Amide*]. L-Cystine bis(L-tyrosine amide) (119 mg) (Vega Chemicals) in 5 mL of 0.05 M borate, pH 8.0, was reduced at room temperature with 44.8 mg of dithiothreitol (Calbiochem). After 2 h, the reaction pH was adjusted to 3 with glacial acetic acid. The reducing agent was removed by chromatography on Sephadex G-10 in 0.1 M acetic acid, and peptide-containing fractions were pooled and lyophilized. The lyophilysate was dissolved in 0.1 M citrate, pH 6.2, and 25 mg of 3-(2-iodoacetamido)-2,2,5,5-tetramethylpyrrolidinyl-1-oxy (Syva) was added. Ethanol was added to the reaction mixture until the spin-label dissolved; the final ethanol concentration was ~15%. The reaction solution was stirred at room temperature for 2 h and the reaction terminated by adjusting to pH 3 with glacial acetic acid. The products were fractionated by chromatography on Sephadex G-10 eluted with 0.1 M acetic acid. The unfractionated products contained a small percentage of the parent disulfide and both the desired spin-labeled peptide and its hydroxylamine reduction product. Fractions containing spin-labeled peptide (which had a lower mobility than the parent disulfide and a slightly greater mobility than the hydroxylamine derivative) were identified by a combination of thin-layer chromatography (see below), UV absorbance, and ESR spectroscopy and were pooled and lyophilized. The final product behaved as a single component on TLC with a trace contaminant visible only under conditions of heavy loading. Upon hydrolysis and amino acid analysis, carboxymethylcysteine and tyrosine were found in a ratio within experimental error of 1:1.

General Methods. For amino acid analyses, the peptides were first hydrolyzed in 6 N HCl in vacuo at 110 °C for 24 h; the composition of the hydrolysate was determined with a Durrum automated amino acid analyzer. Thin-layer chromatography was carried out with plates precoated with silica gel and fluorescent indicator UV_{254} (Brinkmann Instruments). The standard solvent system was 1-butanol–acetic acid–water (9:1:2.5 v/v/v); spots were visualized under UV light and by reaction with ninhydrin spray. ESR studies were carried out as previously described (Lundt & Breslow, 1976).

Results

Development of a Synthesis for 4-(*Glycyl*-L-*phenylalanylamido*)*-2,2,6,6-tetramethylpiperidinyl-1-oxy.* The detailed synthesis of spin-label A is presented under Materials and Methods and is outlined in Figure 1. The choice of the peptide was governed by the known specificity of the principal hormone-binding site which minimally requires peptides containing (1) a free α-amino, (2) an aromatic residue in position 2, and (3) an uncharged carboxyl terminus if the peptide is short [e.g., Breslow et al. (1973)]. In order to maximize exchange rates on the NMR time scale between free and bound states, it was important to use a low association constant (see also Discussion); to achieve this, we chose a minimal side chain in position 1 (Breslow et al., 1973). Placement of the nitroxide on a nonpolar substituent in a position similar to (but not identical with) that of position 3 of bound peptides was feasible because position 3 of bound peptides appears to lie in an apolar pocket of loose specificity [e.g., Breslow (1979)].

Initial synthesis of an analogous peptide spin-label was attempted by coupling the *N*-carbobenzoxy derivative of L-alanyl-L-tyrosine with the nitroxide 4-amino-2,2,6,6-tetramethylpiperidinyl-1-oxy (NH_2-Tempo). However, catalytic hydrogenation and other conditions used to remove the carbobenzoxy protecting group led to reduction of the nitroxide to a complex mixture of products. Accordingly, the coupling step was carried out by using 4-aminotetramethylpiperidine; the secondary amino of the piperidine ring is too hindered to react with the dipeptide, allowing selective coupling at the primary amino. The resultant piperidine tripeptide was deblocked in high yield. The rationale was then to selectively oxidize the piperidine of the deblocked tripeptide product to the nitroxide. However, oxidation conditions necessary for conversion of the piperidine to the nitroxide also oxidized the α-NH_2 group. The α-NH_2 group was therefore protected by citraconylation, and the piperidine was oxidized at this stage to the nitroxide. The citraconyl group was removed at pH 3, a condition under which the nitroxide is stable. The structure of the nitroxide product was confirmed by mass spectrometry, amino acid analysis, and ESR (Materials and Methods). Additionally, proton NMR spectra of the nitroxide and of the piperidine tripeptide were compatible with the assigned structures.

Synthesis of S-[[[*3*-(*2,2,5,5-Tetramethylpyrrolidinyl-1-oxy*)*amino*]*carbonyl*]*methyl*]-L-*cysteine*-L-*tyrosine Amide.* The structure of spin-label B is shown in Figure 2. The nitroxide is placed on an extension of the side chain at position 1 of L-cysteinyl-L-tyrosine amide; this side chain participates in binding [e.g., Breslow et al. (1973)]. The synthetic scheme employed (Materials and Methods) involved reduction of the

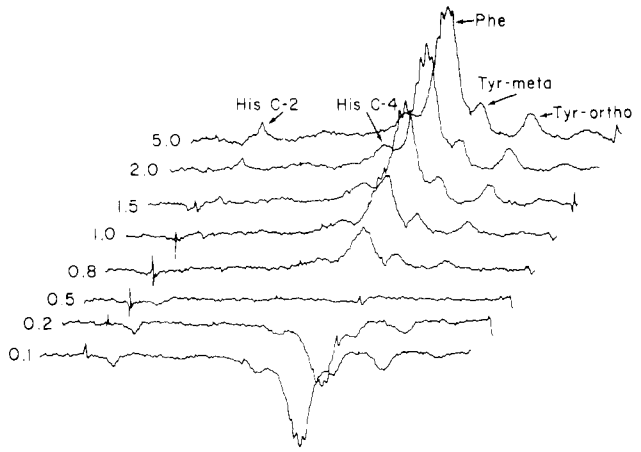


FIGURE 3: Aromatic region of a set of Fourier transform NMR spectra of neurophysin I during an inversion–recovery study. Values at the left are the time in seconds between 180° and 90° pulses. Proton assignments are indicated by arrows.

disulfide of L-cystine bis(L-tyrosine amide) and direct coupling of the isolated sulfhydryl-containing peptide with the nitroxide-containing spin-label. During coupling, reduction products of both the coupled and uncoupled nitroxide were generated but were chromatographically separable from B. The structure of the purified product was confirmed by amino acid analysis and ESR.

T_1 Studies of Spin-Label Effects on Tyr-49 Aromatic Protons of Bovine Neurophysin I. Proton NMR spectra of neurophysin aromatic protons have been previously reported (Cohen et al., 1972; Balaram et al., 1973). Figure 3 shows bovine neurophysin I spectra in the absence of ligands, during a typical inversion–recovery measurement of longitudinal relaxation rate at 220 MHz at pH 6.2. The ortho ring protons of Tyr-49 are well resolved from the meta ring protons and from the ring proton resonances of the three neurophysin phenylalanine residues. Additionally, the meta ring protons of Tyr-49 and the phenylalanine ring protons are well resolved, although downfield shifts of the tyrosine ring protons associated with binding [e.g., Balaram et al. (1973)] lead to somewhat more overlap between the tyrosine meta protons and the phenylalanine ring protons in the bound state.

A series of 11 T_1 studies of the Tyr-49 ortho and meta protons by the inversion–recovery method gave an average T_1 value in the unliganded state of 0.80 ± 0.05 s. A similar set of T_1 studies in the presence of essentially saturating concentrations of the diamagnetic peptide *S*-methyl-L-cysteinyl-L-phenylalanyl-L-isoleucine amide gave a value of 0.75 ± 0.05 s for the liganded state for the ortho protons. Note that this peptide has been shown to bind to the principal hormone-binding site (Breslow et al., 1973) and that its presence at high concentrations does not interfere with observation of the ortho ring protons of Tyr-49 although it does interfere with observation of the meta protons.

In order to determine the distance between the Tyr-49 ring protons and the nitroxides of bound spin-labels, we first measured the effects of increasing spin-label concentrations of the T_1 of Tyr-49 and compared these with the intermolecular relaxation produced by comparable concentrations of nitroxides which do not bind to neurophysin. The nonbinding nitroxides used as controls were NH_2-Tempo and its succinylated derivative S-Tempo (Materials and Methods). ESR studies confirmed that no broadening of the ESR signals of these nitroxides occurred with any of the protein concentrations used; moreover, they do not have the structural features necessary for binding to neurophysin. The nonbinding nitroxides were also compared to the spin-labels with respect to their ability to induce intermolecular relaxation effects. Table I compares the intermolecular effects of A, B, and S-Tempo on the relaxation rate of the tyrosine ring protons of the peptide Ser-Tyr-NH_2. Results indicate that intermolecular effects of A and S-Tempo are comparable while effects of B are 50% greater than those of the others. The origin of the increased intermolecular effect of B is uncertain, but it is small relative to the large effect seen below of B on the neurophysin tyrosine.[2]

Table I: Effects of Nitroxides on the T_1 of the Ring Protons of L-Seryl-L-tyrosine Amide[a]

	$\Delta(1/T_1)$ (s^{-1})/(concn × 10^3)	
	ortho protons	meta protons
S-Tempo	0.38	0.35
spin-label A	0.32	0.34
spin-label B	0.53	0.58

[a] Results are expressed as the difference between $1/T_1$ (s^{-1}) in the absence of nitroxide and that obtained in the presence of nitroxide, divided by the nitroxide concentration.

Table II shows effects of spin-label A, spin label B, and the nonbinding nitroxides on Tyr-49 T_1 values. Results shown include data previously reported (Lord & Breslow, 1978) and additional data. Note that the effects of nonbinding nitroxides have been previously shown to be linearly related to concentration over the concentration range studied (Lord & Breslow, 1978). The results indicate that, over the concentration range 0–1 mM, spin-label A leads at best to a trivial increase in relaxation rate for Tyr-49 protons relative to that induced by nonbinding nitroxides; significant extra relaxation is produced by spin-label B, particularly when the low concentration of B is taken into account.

The minor extra relaxation produced by binding spin-label A leads to the question as to whether A was indeed bound under the conditions employed for NMR studies. Binding of A to neurophysin I in solutions used for NMR studies was confirmed by measuring the ESR signal of A in these solutions at pH 6.2 (where the NMR spectra were obtained and where A should be partially bound) relative to that obtained on the same solutions in the presence of high concentrations of the competing diamagnetic peptide *S*-methyl-L-cysteinyl-L-phenylalanyl-L-isoleucine amide (S-CH_3-CFI); the latter condition represents one in which the strong site is saturated with the diamagnetic peptide and the spin-label should be completely displaced (Breslow et al., 1973; Lundt & Breslow, 1976). Figure 4 shows the ESR data obtained. The sharp signals from the spin-label seen at pH 6.2 in the presence of competing S-CH_3-CFI can be shown to indicate no measurable binding under these conditions, while the more broadened signals seen at pH 6.2 in the absence of competing S-CH_3-CFI indicate that ~50% of the spin-label is bound under the conditions used for T_1 studies in Table II; i.e., the height of the individual resonances in the presence of protein alone at pH 6.2 is 50% the signal height at the same pH in the presence of S-CH_3-CFI. (Note that the contribution of bound spin-label A to peak height is very small at this level of spin-label binding.) The degree of binding observed here in the absence of diamagnetic peptide is identical with that calculable from

[2] The apparently larger intermolecular effect of B on tyrosine protons is not an artifact generated by the "mixing" of the relaxation rates of the rapidly relaxing tyrosine protons of B with those of the tyrosine protons being observed. This can be argued from several observations but is seen most simply from the fact that, at the concentrations of B used for relaxation studies, its own NMR signal is not visible.

Table II: Effects of Spin-Labels and Nonbinding Nitroxides on Tyr-49 T_1 Values at pH 6[a]

NP I[b] concn (mM)	total nitroxide concn (mM)	free nitroxide concn (mM)	$\bar{\nu}_{N\rightarrow O}$	ortho protons		meta protons	
				T_1 (s)	$1/T_1$ (s^{-1})	T_1 (s)	$1/T_1$ (s^{-1})
2.5 → 5			0	0.80 ± 0.05	1.25	0.80 ± 0.05	1.25
5	1 (NH_2-Tempo)	1 (NH_2-Tempo)	0	0.68	1.47		
5	1 (S-Tempo)	1 (S-Tempo)	0	0.69 ± 0.03[c]	1.45	0.67 ± 0.02	1.49
2.9	0.66 (A)	0.37 (A)	0.10	0.69	1.45	0.65	1.54
4.6	0.95 (A)	0.45 (A)	0.11	0.66 ± 0.01	1.51	0.67	1.49
3.8	1.03 (A)	0.53 (A)	0.13	0.625 ± 0.025	1.60	0.61	1.64
2.5	1.14 (A)	0.74 (A)	0.16	0.64	1.56	0.55	1.82
3.5	0.2 (B)	0.022 (B)	0.05	0.61	1.64	0.55	1.82
3.5	0.4 (B)	0.05 (B)	0.10	0.40 ± 0	2.5	0.50 ± 0.01	2.02

[a] Results are averages of several experiments where standard deviations are given. Otherwise each T_1 was obtained from a single experiment. Values of $\bar{\nu}$ (moles of spin-label bound per 10 000 grams of neurophysin) were calculated by using previously determined binding constants (Lundt & Breslow, 1976). [b] NP I, neurophysin I. [c] Results were obtained from studies of the effect of increasing S-Tempo concentrations on T_1 values (Lord & Breslow, 1978) and corrected to allow for an uncertainty of 0.02 s in control values (no spin-label) in this study. Effects of S-Tempo on $1/T_1$ were linearly related to concentration over the concentration range studied (0–1.2 mM).

Table III: Effects of Diamagnetic Peptide on Nitroxide-Induced Longitudinal Relaxation of Tyr-49

additions (total concn)	$\bar{\nu}_{spin\text{-}label}$[a]	$\bar{\nu}_{S\text{-}CH_3\text{-}CFI}$[b]	obsd		effects of nitroxide addition, $\Delta(1/T_1)$ (s^{-1})
			T_1 (s)	$1/T_1$ (s^{-1})	
NP I			0.80	1.25	
+S-Tempo (1 mM)			0.69	1.45	+0.20
+A (1 mM)	0.12		0.64	1.56	+0.31
+B (0.4 mM)	0.10		0.40	2.5	+1.25
+S-CH_3-CFI (9 mM)		0.96	0.75	1.33	
+S-CH_3-CFI (9 mM) + S-Tempo (1 mM)		0.96	0.65	1.54	+0.21
+S-CH_3-CFI (9 mM) + A (1 mM)	0.015	0.96	0.44	2.27	+0.94
+S-CH_3-CFI (9 mM) + B (0.4 mM)	0.023	0.94	0.43	2.33	+1.00

[a] Calculated fractional occupancy of the strong binding site by spin-label. [b] Calculated fractional occupancy of the strong binding site by S-CH_3-CFI.

the previously determined binding constant for spin-label A, 2.7×10^2 M^{-1} (Lundt & Breslow, 1976).

Effects of Diamagnetic Peptides on Tyr-49 T_1 Values in the Presence of Spin-Labels. Under conditions in which A is visibly displaced from the protein by diamagnetic peptide (see Figure 4) relaxation of Tyr-49 by A would be predicted to be identical with that induced by nonbinding nitroxides. Table III shows that this is not the case. This table reports the effects of spin-label and nonbinding nitroxides when added to neurophysin in the presence of concentrations of S-CH_3-CFI sufficient to replace virtually all of the bound spin-label at the principal peptide-binding site. Specifically, by use of known binding constants at pH 6.2 for S-CH_3-CFI (Breslow et al., 1973) and the spin-labels (Lundt & Breslow, 1976), it can be calculated that, in the presence of S-CH_3-CFI, the known peptide-binding site can be occupied to an extent no greater than 2% by spin-label A or 3% by spin-label B. The data in Table III indicate the following. First, the nonbinding nitroxide S-Tempo has the same effect on the T_1 of Tyr-49 whether it is added in the presence or absence of S-CH_3-CFI. Second and most significantly, despite the lack of effect of S-CH_3-CFI on the T_1 effects of nonbinding nitroxides, the presence of S-CH_3-CFI leads to a marked increase in the T_1 effects of spin-label A and to only a small reduction of the T_1 effect of spin-label B, although most of B and A are displaced from the peptide-binding site by the diamagnetic peptide.

The unexpected effects of diamagnetic peptide on T_1 relaxation by A and B have been interpreted in terms of a second very weak binding site immediately adjacent to Tyr-49 (Lord & Breslow, 1978) to which the spin-labels are displaced when the strong site is occupied by diamagnetic peptide. It is relevant to emphasize that this second site appears to have some specificity attributes in common with the strong site since A and B bind to it but NH_2-Tempo and S-Tempo do not.

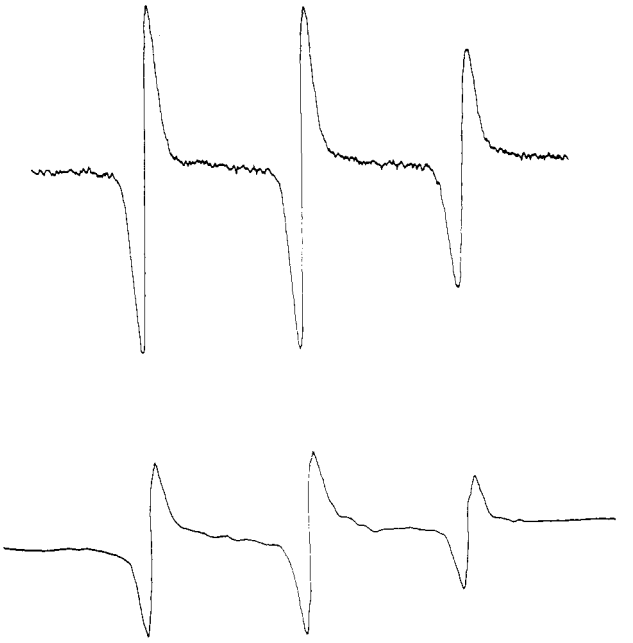


FIGURE 4: ESR spectra of spin-label A in the presence of neurophysin I. (Lower spectrum) 1 mM spin-label A plus 5 mM neurophysin I, pH 6.2. (Upper spectrum) Same sample as in lower spectrum following addition of 9 mM S-CH_3-CFI. Note that lower spectrum was redrawn from the original so that both spectra would reflect the same scale expansion.

T_2 Studies of Spin-Label Effects on Tyr-49. The effects of spin-labels, diamagnetic peptides, and nonbinding nitroxides on the T_2 of the ortho ring protons of Tyr-49 were determined

Table IV: Representative Line Width Studies of Ortho Ring Protons of Tyr-49[a]

conditions	$\bar{\nu}_{\text{spin-label}}$	line width (Hz)	T_2' (s)	$1/T_2'$ (s^{-1})
NP I[b]	0	21.5	0.0148	67.5
NP I + S-Tempo (1 mM)	0	22.5	0.0141	70.7
NP I (4.6 mM) + A (0.95 mM)	0.11	22.9	0.0139	71.9
NP I (3.8 mM) + A (1 mM)	0.13	23	0.0138	72.2
NP I (2.5 mM) + A (1.1 mM)	0.16	23	0.0138	72.2
NP I (3.5 mM) + B (0.2 mM)	0.05	27.7	0.0115	87
NP I (3.5 mM) + B (0.4 mM)	0.10	36	0.0088	113
NP I + S-CH_3-CFI (9 mM)	0	23.4	0.0136	73.5
NP I + S-CH_3-CFI (9 mM) + S-Tempo (1 mM)	0	24	0.0133	75.4
NP I + S-CH_3-CFI (9 mM) + A (1 mM)	0.015	30	0.0106	94.2
NP I + S-CH_3-CFI (9 mM) + B (0.4 mM)	0.023	28.6	0.0111	89.8

[a] Line width at peak half-height is used to determine an apparent T_2 (T_2') according to the relationship $T_2' = 1/(\pi\Delta\nu_{1/2})$. [b] NP I, neurophysin I.

Table V: Distance Calculations between Tyr-49 and N → O of A and B

obsd nucleus	spin-label	T_{1mpc} (s)	T_{2mpc} (s)	τ_c (s)	r (Å)	τ_c (s)	r (Å)
ortho protons	A	0.8[a]	0.05[a]	3.3×10^{-9}	14.5	4×10^{-9} [c]	14.0
		1.7[b]	0.2[b]	2.2×10^{-9}	17.3	4×10^{-9} [c]	15.7
meta protons	A	0.6[a]	not calcd	3.3×10^{-9} [c]	13.8	4×10^{-9} [c]	13.3
		0.8[b]	not calcd	2.2×10^{-9} [c]	15.1	4×10^{-9} [c]	14.0
ortho protons	B	0.1[a]	0.0024	5.7×10^{-9}	9.5	4×10^{-9} [c]	9.8
		0.11[b]	0.0025	5.7×10^{-9}	9.6	4×10^{-9} [c]	9.9
meta protons	B	0.11[a]	not calcd	5.7×10^{-9} [c]	9.6	4×10^{-9} [c]	9.9
		0.12[b]	not calcd	5.7×10^{-9} [c]	9.7	4×10^{-9} [c]	10.1

[a] Calculated by assuming that only free spin-label relaxes by intermolecular mechanism. [b] Calculated by assuming equivalent intermolecular relaxation by bound and free spin-label. [c] Assumed values; all other values of τ_c are calculated as described in the text.

from line width measurements (Table IV). In discussing Table IV, it is relevant to note that the width at half-height of the Tyr-49 ortho proton peak is not a direct measure of T_2; this width contains the coupling constant which, in the free amino acid, divides the ortho protons into two peaks ~9 Hz apart and also contains potential contributions from environmental nonequivalence of the two ortho protons. Nonetheless, the paramagnetic molecule-induced *changes* in line width are a measure of paramagnetic contributions to T_2 since the other contributions are subtracted out. To reflect the multiple contributions to line width, in Table IV we used the term T_2'; this is defined by the relation $T_2' = 1/(\pi\Delta\nu_{1/2})$, where $\Delta\nu_{1/2}$ is the line width of the tyrosine proton peak at peak half-height. The results in Table IV exactly parallel the T_1 effects; i.e., line broadening by A alone is minimal, line broadening effects of B greatly exceed those of A, line broadening by A is greatly increased in the presence of S-CH_3-CFI, and line broadening by B is only slightly reduced by S-CH_3-CFI. These results confirm both the relative effects of the different spin-labels and the conclusion previously obtained from T_1 data supporting the presence of a very weak second spin-label binding site immediately adjacent to Tyr-49.

Effects of Spin-Labels on Other Residues. In Figure 3, the C-2 and C-4 protons of the single neurophysin I histidine (position 80) are also seen. The C-2 proton is particularly well separated from the other aromatic protons, but the shapes of both proton peaks are less distinct than optimal. In the absence of S-CH_3-CFI no systematic significant effect of either spin-label A or B on the C-2 or C-4 protons was seen. In the presence of saturating concentrations of S-CH_3-CFI, spin-label A led to a slight decrease in T_1 of the C-2 proton (the C-4 cannot be observed under these conditions), but the experimental significance of this is uncertain.

Both A and B produced the same moderate decrease in the average T_1 of the three protein phenylalanine residues at comparable levels of binding. As previously reported (Lord & Breslow, 1979), spin-label A led to a decisive broadening of the methyl protons of the amino-terminal alanine in the absence, but not in the presence, of S-CH_3-CFI, indicating that the broadening resulted from binding of A to the strong site.

Distance Calculations. The salient features of the calculations we have used for distance determinations are presented in this section. Details are given in the Appendix. First, we have assumed that the interactions of both spin-labels A and B with the principal hormone-binding site of neurophysin are fast on the T_1 time scale. This assumption is justified on the basis of the kinetics of interaction of other peptides with neurophysin (Pearlmutter & McMains, 1977; Pearlmutter & Dalton, 1980; Appendix) and allows the data to be treated by fast-exchange equations (Appendix) to generate the intracomplex paramagnetic contribution of bound spin-label to the longitudinal relaxation time of the tyrosine protons (T_{1mpc}). The value of T_{1mpc} in turn can be used to derive the distance between the nitroxide and observed proton if the correlation time governing the dipolar interaction between the nitroxide and the proton is calculated. To calculate the correlation time, we have also assumed that spin-proton interactions are fast on the T_2 time scale; this appears readily justifiable for spin-label A (Appendix) although it is less certain to be correct for spin-label B for the conditions used here (Appendix and Discussion). Fast-exchange conditions on the T_2 time scale allow the intracomplex paramagnetic contribution of bound spin-label to the transverse relaxation time of the observed proton (T_{2mpc}) to be calculated. The ratio T_{1mpc}/T_{2mpc} is used to calculate the correlation time (Appendix).

Table V shows values of T_{1mpc}, T_{2mpc}, and correlation time (τ_c) calculated for the two spin-labels and different tyrosine protons. For each set of observations, two values of each of these parameters are given, reflecting an ambiguity in estimating and correcting for the *inter*molecular effects of bound spin-label; i.e., a fraction of the total spin-label is bound by the protein (Table II) and may lead to intermolecular relaxation effects (effects on protein molecules to which they are

not bound) different from those generated by unbound nitroxides (Krugh, 1976). Our data suggest (Appendix) that binding does not increase the intermolecular relaxation contributions of these spin-labels. Accordingly, in Table V, results are calculated by assuming in one case that both free and bound spin-label are equivalent to S-Tempo in their intermolecular relaxation effects and, in the other, that only unbound spin-label generates significant intermolecular relaxation. Values of τ_c in Table V are in good agreement with the value of 4×10^{-9} s calculated for the rotational correlation time of hormone–neurophysin complexes at low pH by Deslauriers et al. (1979) but are significantly lower than several other estimates of the rotational correlation time [e.g., Alazard et al. (1974)]. We have also calculated a value for τ_c of 4.5×10^{-9} s from the T_1 and T_2 values obtained in the presence of spin-label and S-CH_3-CFI.

Column 6 of Table V shows the distances calculated for each set of observations from the calculated values of T_{1mpc} and τ_c. Because it is not certain that apparent τ_c differences for the different spin-labels are real or that T_{2mpc} values (and hence τ_c values) for B are reliable (vide supra), distance values were also calculated for each spin-label by using a value of 4×10^{-9} s for τ_c (Table V, last column). In an earlier study (Lord & Breslow, 1979), we estimated the distance between the nitroxide of spin-label A bound to the strong site and the methyl resonances of the amino-terminal alanine of neurophysin I by using the line broadening of the alanine methyl resonances by A to obtain T_{2mpc} and an estimated τ_c value of 1×10^{-8} s. For a value of $\tau_c = 4 \times 10^{-9}$ s, the earlier value of r should be revised downward to 12.2 Å. In Figure 5, our estimates of all spin-label to tyrosine distances are shown, together with the estimated distance between spin-label A and the amino terminus. The model used to describe the conformation of the protein in Figure 5 is largely hypothetical but is meant to emphasize a central role for the highly conserved residues 10–77 (shown in the figure in darker outline) and to suggest separation of the duplicated segments into individual domains. Neurophysin is dimeric under the conditions of this study (Nicolas et al., 1978) and the model additionally assumes that distances calculated are all within the same subunit. The significance of Figure 5 will be discussed further below.

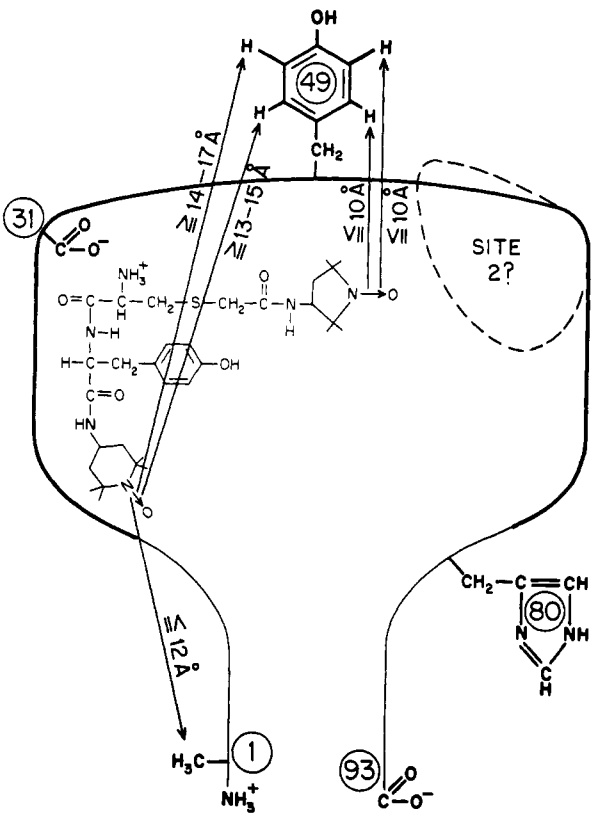


FIGURE 5: Hypothetical model of neurophysin I showing distances calculated between spin-label bound to the strong site and individual protein protons. Circled numbers refer to protein residues. The region of the protein drawn in heavier outline represents residues 10–77. Bound spin-label is shown as a composite of spin-labels A and B. The suggested second site location is intended only to apply to the N → O group(s) of spin-labels bound to this site. Details of folding within individual segments of the protein and of disulfide pairing are omitted in this schematic representation.

Discussion

Secondary-Binding Sites for Peptides. The markedly increased relaxation rates of Tyr-49 ring protons when spin-label A is displaced from its strong binding site by diamagnetic peptide are best explained by the assumption of secondary binding sites for spin-label that are immediately adjacent to Tyr-49 (Lord & Breslow, 1978).[3] This conclusion parallels that of Cohen and co-workers (Nicholas et al., 1978) who found that secondary binding sites for the hormones were blocked when Tyr-49 was nitrated. However, all our efforts to detect secondary sites for small peptides by thermodynamic measurements [e.g., Lundt (1977)] indicate that they can have an affinity no greater than $^1/_{100}$ that of the principal peptide-binding sites. Additionally (Bothner-By et al., 1980), we have been unable to detect secondary hormone-binding sites of the affinity reported by others [e.g., Nicolas et al. (1978)]. While it is therefore probable that secondary sites, particularly for small peptides, are extremely weak, these sites are detectable in the present studies by virtue of their fortuitously close proximity to Tyr-49 and the r^6 dependence of spin-label effects. The presence of two classes of peptide-binding sites with similarities in specificity (see Results) supports the suggestion that the duplicated segments may each provide a binding site under appropriate conditions (Camier et al., 1973). However, in view of the large affinity disparity between the two classes of sites, confirmation of this hypothesis awaits further study.

Limitations of Distance Estimates. Several factors potentially affecting the validity of our distance estimates are relevant. The most general of these is the potential influence of spin diffusion on T_1 values [e.g., Sykes et al. (1978)]. Although contributions of spin diffusion to T_1 values in the diamagnetic state are automatically subtracted, the question arises as to whether spin diffusion contributes to spin-label induced changes in T_1. Spin diffusion would be expected to offer alternate modes of relaxation for each nucleus through

[3] An alternative explanation for these results can be formulated but is not quantitatively compatible with the data. Neurophysin is dimeric in these studies (see text). In the absence of S-CH_3-CFI, most spin-label is bound to dimer in which only one chain is in the liganded state, while in the presence of S-CH_3-CFI all spin-label is bound to molecules in which both chains are liganded. If it is assumed that the conformations of the half-liganded and fully liganded dimers differ such that the strong site is closer to Tyr-49 in the fully liganded state, then an enhancement of spin-label relaxation by S-CH_3-CFI might be qualitatively explained without invoking a second site per chain. However, using known peptide and spin-label binding constants (see text) and considering demonstrated cooperative interactions between the two strong sites of the dimer [e.g., Breslow & Gargiulo (1977)], we calculate that, because of competition from S-CH_3-CFI, the *absolute number* of spin-label molecules bound to a strong site of a fully liganded dimer in the absence of S-CH_3-CFI is not significantly different from that in the presence of S-CH_3-CFI; the very large effects of S-CH_3-CFI on relaxation by A are therefore not explained.

its effects on adjacent nuclei. Thus, the observed paramagnetic relaxation enhancements might in part reflect effects on nuclei adjacent to those under observation and lead to underestimates in r. (Note that underestimates in T_{1mpc} affect r both directly and through their effects on estimates of τ_c, but the overall effect is to generate an underestimate in r.) We consider the problem to be relatively unimportant here because of the internal consistency of T_2 effects and T_1 effects and because significant differences between different spin-labels and protons were seen; e.g., the major Tyr-49 relaxation rate enhancements generated by B, or by A in the presence of S-CH_3-CFI, appeared to be discrete and not distributed over a large number of protein resonances. We do not, however, exclude the possibility that very small spin-diffusion effects represent a significant contribution to the small relaxation rate enhancement of Tyr-49 protons by A in the absence of S-CH_3-CFI.

A second factor is the potential effect of weak site occupancy on distance calculations involving the strong site. The weak binding site adjacent to Tyr-49 is principally observed when the strong site is occupied with S-CH_3-CFI. To what extent might the weak site be occupied in the absence of diamagnetic peptide and therefore be partially responsible for spin-label relaxation of Tyr-49 under these conditions? We have previously presented data suggesting that apparent occupancy of the weak site, as measured by Tyr-49 effects, depends on free spin-label concentration and total primary site occupancy (Lord & Breslow, 1978). On the basis of this, we calculate (Appendix) that 40% of the Tyr-49 relaxation induced by A in the absence of S-CH_3-CFI might arise from weak site occupancy, which in turn suggests that calculated distances between A at the strong site and Tyr-49 are underestimates by $\sim$10%. Similar calculations for B, however, indicate that weak site occupancy in the absence of S-CH_3-CFI should have no significant effect on these strong site distance calculations. Also, secondary site occupancy should have no effect on distance calculations involving the amino terminus since the paramagnetic contribution to relaxation of these nuclei by A is only present when A is bound to the strong site (vide supra).

While the above considerations suggest that calculated distances between spin-label A at the strong site and Tyr-49 may be underestimates, distances between B at the strong site and Tyr-49 may be overestimates. The uncertainty with respect to B arises from the possibility that B is not in strict fast exchange on this time scale. If this is so, then τ_c for this interaction is greater than calculated and r is less than calculated. Note that we do not consider it unreasonable that the value of τ_c for the different spin-labels may be nonidentical since the relative mobility of the different nitroxides on the protein surface may be nonidentical. In this context, it is also relevant to note that, since the amino terminus appears to be a region of particularly high mobility (Lord & Breslow, 1979), then the real value of τ_c for this region may be shorter than the value of 4×10^{-9} s used above and that, accordingly, spin-label A may be closer to the amino terminus than shown in Figure 5.

Implications of Data for Intertyrosine Distances. Our results (Table V) and the arguments above indicate that the distance between the nitroxide of A at the strong site and the ortho protons of Tyr-49 is >14 Å. In order to use this distance to calculate the minimum distance between the aromatic residue (Tyr or Phe) in position 2 of bound peptides and the ortho protons of Tyr-49, we must use the distance between position 2 and the nitroxide (taken as the center of the N $\rightarrow$ O bond) in the bound state. Model studies indicate that this distance must be between 12.8 Å (fully extended peptide) and 4 Å (compactly folded peptide). Use of the average distance, 8.4 Å, leads to the conclusion that the ortho protons of Tyr-49 and the ortho protons of position 2 of the peptide must be $\geq$5.6 Å apart, in agreement with the low level of fluorescence energy transfer between Tyr-49 and position 2 (Sur et al., 1979). This distance suggests that the previously observed nuclear Overhauser effect between the ortho protons of Tyr-49 and the ortho protons of the peptide ring in position 2 may have been generated over a relatively long distance via spin diffusion at the strong site and/or that it arises from peptide at the weak site. We tentatively consider it unlikely that the weak site is the major NOE contributor. The NOE between peptide ring protons in position 2 and aromatic protons of Tyr-49 was retained when Tyr-49 was nitrated (Balaram et al., 1973); as cited above, nitration is reported to block binding to the weak site. It must also be pointed out, however, that strong site intertyrosine distances shorter that 5.6 Å are possible *if* the peptide is rigidly bound in an extended conformation. There is no evidence which allows this possibility to be strictly excluded.

Implications of Data for the Location of the Strong Binding Site. Affinity labeling studies (Walter & Hoffman, 1973) suggest that the α-amino group of hormones bound to the strong site interacts with Glu-31, the carboxyl-terminal residue of the first duplicated segment. The model in Figure 5 utilizes this observation and the distances obtained from spin-label studies to orient peptides bound to the strong site, relative to the amino-terminal half of the protein, with the extended side chain of peptide residue 1 situated in space between Glu-31 and Tyr-49 and the side chain of residue 3 oriented toward the amino terminus. The data do not preclude the participation of residues other than 12–31 in the strong binding site, and such participation might be suggested by the strong evolutionary conservation of residues 10–77. However, the results argue against direct participation of either Tyr-49 or His-80 at the strong site; the lack of involvement of His-80 at the strong site is consistent with other data [e.g., Lundt & Breslow (1976)].

We have previously suggested that both duplicated regions might participate together at the strong site while behaving separately in weak site interactions (Breslow, 1979). It is relevant to extend this suggestion in the light of Figure 5 and the ligand-facilitated dimerization of neurophysin (Nicolas et al., 1978). The general structure in Figure 5 is similar to the crystallographically determined structure of wheat germ agglutinin (Wright, 1977),[4] which also dimerizes. If the dimerization modes of the two proteins are alike, then, in the neurophysin dimer, the amino-terminal half of one subunit would be proximal to the carboxyl-terminal half of the other subunit. Neurophysin dimerization is strengthened by binding of peptide to the strong site. Such a situation might be explained if the interactions of bound peptide with the amino-terminal duplicated domain of one subunit were supported by weak interactions with spatially proximal residues from the carboxyl-terminal duplicated domain of the other subunit.

Conclusions. The present study demonstrates that spin-labels capable of binding to the hormone-binding site(s) of neurophysin can be synthesized and used to explore distances between these sites and individual protein residues. Preliminary distance estimates have been made for Tyr-49, the amino terminus, and His-80 of bovine neurophysin I. These studies

[4] The possibility that the conformations of neurophysin and wheat germ agglutinin might be similar was suggested to us by Dr. A. Wright.

are extendable in principle to bovine neurophysin II in which the resonances of position 1, 2, and 49 have been identified (Lord & Breslow, 1979) and to neurophysins selectively modified to permit identification of other resonances.

Acknowledgments

We are particularly grateful to Rockefeller University for generously providing NMR spectrometer time and for mass spectral analyses and to the Middle Atlantic NMR Facility where some of our supporting studies were performed. We also acknowledge the assistance of both the Department of Chemistry of Columbia University and Dr. F. Landsberger of Rockefeller University in providing the EPR spectrometer time essential to these studies.

Appendix

Equations and Assumptions Used in Distance Calculations. Arguments for fast interactions on an NMR time scale between spin-label A and the strong hormone-binding site of neurophysin are illustrated by comparing the expected exchange rate of spin-label A with the requirements for fast exchange specified by Wien et al. (1972). T_2 measurements are chosen for this comparison because $T_2 \ll T_1$ in our systems; therefore, systems for which exchange is fast relative to T_2 will show fast exchange relative to T_1. According to Wien et al. (1972), the conditions for fast exchange are satisfied when

$$\tau_M \ll T_{2M} \qquad \tau_A \ll T_{2A} \tag{1}$$

and

$$\frac{f_A}{T_{2A}} + \frac{f_M}{T_{2M}} \gg \tau f_A^2 f_M^2 (\omega_A - \omega_M)^2 \tag{2}$$

Here T_{2M} and T_{2A} are the transverse relaxation times of the observed nucleus when it is in the paramagnetic (bound state) and diamagnetic environments (free state), respectively, f_M and f_A are the fraction of the nuclei in the paramagnetic and diamagnetic environments, respectively, τ_M and τ_A are the lifetimes of the nucleus in the paramagnetic and diamagnetic environments, respectively, and $\tau = \tau_M + \tau_A$; ω_M and ω_A are the chemical shifts of the nucleus in the paramagnetic and diamagnetic environments, respectively, and differ from each other by 0.1 ppm (Balaram et al., 1973). The kinetics of neurophysin interaction with spin-labels have not been measured per se. However, recent kinetic studies by Pearlmutter & Dalton (1980) indicate that affinity differences between the hormones and several small peptides for the principal hormone-binding site are largely a reflection of differences in dissociation rate of the complexes. This observation is supported by NMR exchange rate differences between the "tighter" hormone–neurophysin complexes which exchange slowly and the "looser" peptide–neurophysin complexes which exchange rapidly (Balaram et al., 1973). Accordingly, it is likely that the 700-fold weaker binding of spin-label A compared to that of the hormones [cf. Breslow et al. (1973) and Lundt & Breslow (1976)] is reflected in an ~700-fold faster dissociation constant or a rate ($1/\tau_M$) of ~7000/s; the "on rate" ($1/\tau_a$) can be estimated under the conditions used as ~1000/s. Comparison of these values with values of $1/T_2'$ (which places an upper limit on $1/T_2$) or $1/T_{2mpc}$ for spin-label A, ~70/s and <20/s, respectively, indicates that fast-exchange conditions are met. For spin-label B, exchange can similarly be argued to be fast on the T_1 time scale, but fast exchange may not prevail on the T_2 time scale. This situation arises in part because of the very short relaxation time (Table V) estimated for Tyr-49 ring protons when B is bound; this value (~0.0025 s) is only slightly greater than reasonable estimates (based as above on binding constants) of τ_M for B (~0.001 s).

For a spin-label system in fast exchange, the observed relaxation rate, $1/T_{obsd}$, is the sum of the relaxation rate of the nucleus in its different environments as indicated by eq 3. As

$$\frac{1}{T_{obsd}} = \frac{f_A}{T_A} + \frac{f_M}{T_M} \tag{3}$$

discussed with particular thoroughness by Krugh (1976), there are paramagnetic and diamagnetic contributions both to T_A and T_M. Thus, the total relaxation rate in the unbound state ($1/T_A$) is the sum of that of the unperturbed nucleus in the unbound state ($1/T_a$) plus the intermolecular paramagnetic relaxation rate enhancements ($1/T_{ap}$) induced by nitroxides either free in solution or bound to different molecules. The total relaxation rate in the bound state ($1/T_M$) is the sum of that of the nucleus in the bound state in the absence of paramagnetic species ($1/T_m$), plus the intermolecular paramagnetic relaxation rate enhancement ($1/T_{mp}$) induced by nitroxides either free in solution or bound to different molecules, plus the *intracomplex* paramagnetic relaxation rate enhancement ($1/T_{mpc}$) produced by spin-label bound to the molecule the nucleus of which is being observed. Therefore

$$\frac{1}{T_{obsd}} = \frac{f_A}{T_a} + \frac{f_A}{T_{ap}} + \frac{f_M}{T_m} + \frac{f_M}{T_{mp}} + \frac{f_M}{T_{mpc}} \tag{4}$$

For distance calculations, the term to be obtained from the data is T_{mpc}, since the distance between the bound nitroxide and the nucleus under observation, at a 220-MHz frequency, is (for macromolecular systems in which hyperfine terms are neglectable)

$$r(\text{Å}) = 540\left[\left[\frac{3\tau_c}{1 + (13.8 \times 10^8)^2\tau_c^2}\right]T_{1mpc}\right]^{1/6} = 480\left[\left[4\tau_c + \frac{3\tau_c}{1 + (13.8 \times 10^8)^2\tau_c^2}\right]T_{2mpc}\right]^{1/6} \tag{5}$$

where τ_c is the correlation time that governs the interaction between the nucleus and the bound spin-label [e.g., Krugh (1976)]. All terms other than T_{mpc} in eq 4 are known from the data, and, therefore, T_{mpc} can be calculated. T_a and T_m are the relaxation times in the free protein and in the protein–CFI complex, respectively (in the absence of paramagnetic species), and T_{ap} and T_{mp} are obtained from the effects of S-Tempo on the free protein and protein–CFI complex, respectively. The only uncertainty results from the uncertainty as to whether the intermolecular relaxation effects of free and bound spin-label are equivalent (see Results). From the lack of effect of both spin-labels on the histidine protons and the trivial differences between A and S-Tempo on Tyr-49 in the absence of S-CH_3-CFI, we assume that binding does not significantly increase the intermolecular relaxation contributions of these spin-labels, but a decrease in their intermolecular relaxation contributions on binding cannot be excluded. The treatment of the data (Results) reflects this ambiguity.

In addition to T_{mpc}, a value of τ_c must be determined before r can be calculated (eq 5). In principle, τ_c is a function of the motional characteristics (τ_r) of the nucleus and bound spin-label, of the electron spin relaxation time (τ_s), and of τ_m, being dominated by the shortest of these times. τ_r probably lies between 10^{-9} s (Lundt, 1977) and 5×10^{-8} s (Alazard et al., 1974), τ_m is $\geq 10^{-4}$ s (vide supra), and τ_s is $\sim 10^{-7}$ s (Wien et al., 1972); therefore, τ_c should be determined largely by τ_r.

For our calculations, τ_c was estimated from the ratio T_{1mpc}/T_{2mpc} according to eq 6 [e.g., James (1975)]. The values of

$$\tau_c^2 = \frac{6(T_{1mpc}/T_{2mpc}) - 7}{4(13.8 \times 10^8)^2} \quad (6)$$

τ_c calculated ($\sim 4 \times 10^{-9}$ s) confirm that τ_c is determined by τ_r.

Secondary Site Occupancy Calculations. From previous studies (Lord & Breslow, 1978), the fractional occupancy of the weak site by spin label, $\bar{\nu}_w$, can be defined by eq 7 where

$$\bar{\nu}_w = \frac{\bar{\nu}_s K_w(\text{free spin-label})}{1 + \bar{\nu}_s K_w(\text{free spin-label})} \quad (7)$$

$\bar{\nu}_s$ is the fractional occupancy of the strong site (summed over all bound peptides) and K_w is the binding constant of spin-label to the weak site. Because K_w is very small (vide supra), eq 7 reduces to eq 8. The real value of K_w is not known but is

$$\bar{\nu}_w = \bar{\nu}_s K_w(\text{free spin-label}) \quad (8)$$

assumed to be unaffected by the presence of competing diamagnetic peptide because concentrations of diamagnetic peptide are low relative to weak site affinity (Lord & Breslow, 1978). Moreover, free spin-label concentrations and values of $\bar{\nu}_s$ in the absence and presence of diamagnetic peptide (typically ~ 0.1 and 1.0, respectively) are known. Relaxation $[\Delta(1/T)]$ by spin-label bound to the weak site will be proportional to $\bar{\nu}_w$ and, in the presence of S-CH_3-CFI, is obtained from the data in Table III. Therefore, the relation

$$\frac{\Delta(1/T)_{\text{absence of S-CH}_3\text{-CFI}}}{\Delta(1/T)_{\text{presence of S-CH}_3\text{-CFI}}} = \frac{\sim 0.1(\text{free spin-label})_{\text{absence of S-CH}_3\text{-CFI}}}{1(\text{free spin-label})_{\text{presence of S-CH}_3\text{-CFI}}} \quad (9)$$

will generate weak site occupancy effects in the absence of S-CH_3-CFI. Application of this equation to individual studies indicates that weak site occupancy should, on the average, contribute 40% of the observed relaxation by A in the absence of S-CH_3-CFI but only 1% of the observed relaxation by B in the absence of S-CH_3-CFI.

References

Alazard, R., Cohen, P., Cohen, J. S., & Griffin, J. H. (1974) *J. Biol. Chem. 249*, 6895.

Balaram, P., Bothner-By, A. A., & Breslow, E. (1973) *Biochemistry 12*, 4695.

Bothner-By, A. A., & Johner, P. M. (1977) *Proc. Colloq. Spectrosc. Int. Invited Lect., 20th*, 355.

Bothner-By, A. A., LeMarie, B., Walter, R., Co, R., Rabbani, L. D., & Breslow, E. (1980) *Int. J. Pept. Protein Res.* (in press).

Breslow, E. (1979) *Annu. Rev. Biochem. 48*, 251.

Breslow, E., & Weis, J. (1972) *Biochemistry 11*, 3474.

Breslow, E., & Gargiulo, P. (1977) *Biochemistry 16*, 3397.

Breslow, E., Aanning, H. L., Abrash, L., & Schmir, M. (1971) *J. Biol. Chem. 246*, 5179.

Breslow, E., Weis, J., & Menendez-Botet, C. J. (1973) *Biochemistry 12*, 4644.

Camier, M., Alazard, R., Cohen, P., Pradelles, P., Morgat, J. L., & Fromageot, P. (1973) *Eur. J. Biochem. 32*, 207.

Capra, J. M., Kotelchuck, D., Walter, R., & Breslow, E. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 431.

Chaiken, I. M., Randolph, R. E., & Taylor, H. C. (1975) *Ann. N.Y. Acad. Sci. 248*, 442.

Cohen, P., Griffin, J. H., Camier, M., Caizergues, M., Fromageot, P., & Cohen, J. S. (1972) *FEBS Lett. 25*, 282.

Cohen, P., Camier, M., Wolff, J., Alazard, R., Cohen, J. S., & Griffin, J. H. (1975) *Ann. N.Y. Acad. Sci. 248*, 463.

Deslauriers, R., Smith, I. C. P., Stahl, G. L., & Walter, R. (1979) *Int. J. Pept. Protein Res. 13*, 78.

Furth, A. J., & Hope, D. B. (1970) *Biochem. J. 116*, 545.

Griffin, J. H., Alazard, R., & Cohen, P. (1973) *J. Biol. Chem. 248*, 7975.

Hruby, V. J., Spatola, A. F., Glasel, J. A., & McKelvy, J. F. (1975) *Ann. N.Y. Acad. Sci. 248*, 451.

James, T. L. (1975) *Nuclear Magnetic Resonance in Biochemistry*, Academic Press, New York.

Konig, N., & Geiger, R. (1970) *Chem. Ber. 103*, 788.

Krugh, T. R. (1976) in *Spin Labeling, Theory and Applications* (Berliner, L. J., Ed.) p 339, Academic Press, New York.

Lord, S. T., & Breslow, E. (1978) *Biochem. Biophys. Res. Commun. 80*, 63.

Lord, S. T., & Breslow, E. (1979) *Int. J. Pept. Protein Res. 13*, 71.

Lundt, S. L. (1977) Ph.D. Thesis, Cornell University Medical College, New York.

Lundt, S. L., & Breslow, E. (1976) *J. Phys. Chem. 80*, 1123.

Nicolas, P., Camier, M., Dessen, P., & Cohen, P. (1976) *J. Biol. Chem. 251*, 3965.

Nicolas, P., Wolff, J., Camier, M., DiBello, C., & Cohen, P. (1978) *J. Biol. Chem. 253*, 2633.

Pearlmutter, A. F., & McMains, C. (1977) *Biochemistry 16*, 628.

Pearlmutter, A. F., & Dalton, E. J. (1980) *Biochemistry 19*, 3550.

Sur, S. S., Rabbani, L. D., Libman, L., & Breslow, E. (1979) *Biochemistry 18*, 1026.

Sykes, B. D., Hull, W. E., & Snyder, G. H. (1978) *Biophys. J. 21*, 137.

Vold, R. L., Waugh, J. S., Klein, M. P., & Phelps, D. E. (1968) *J. Chem. Phys. 48*, 3831.

Walter, R., Ed. (1975) *Ann. N.Y. Acad. Sci. 248*, 1–512.

Walter, R., & Hoffman, P. L. (1973) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 32*, 567 (Abstr.).

Weiner, H. (1969) *Biochemistry 8*, 526.

Wien, R. W., Morrisett, J. D., & McConnell, H. M. (1972) *Biochemistry 11*, 3707.

Wright, C. S. (1977) *J. Mol. Biol. 111*, 439.