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# NUCLEAR MAGNETIC RESONANCE SPIN LABEL STUDIES OF NEUROPHYSIN: EVIDENCE FOR SECONDARY PEPTIDE-BINDING SITES*

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SUMMARY

The longitudinal relaxation time, $T_1$, of the ortho ring protons of Tyr-49 of bovine neurophysin-I was investigated in the absence and presence of two spin-labeled peptides which are known to bind to the principal hormone-binding site. Displacement of the spin labels from the principal hormone-binding site by diamagnetic peptide led to anomalous changes in $T_1$. The results suggest the presence of a second binding site for peptides, of low thermodynamic affinity, but immediately adjacent to Tyr-49.

Bovine neurophysins contain a strong site which binds oxytocin or vasopressin and a secondary site, particularly demonstrable for vasopressin, whose relative thermodynamic affinity depends on conditions (1-3). The two sites may reflect the internal duplication of the sequence (4,5). Small peptide analogs of the amino-terminal region of the hormones also bind to neurophysin, but only a single site for these has been observed (5-7); this site appears to correspond to the stronger hormone-binding site (3). Neurophysins each contain a single tyrosine, Tyr-49, in the middle of the peptide chain (4). Nuclear Overhauser and other studies of peptide-binding have suggested a proximity between Tyr-49 and the peptide site (3,8). However, nitration of Tyr-49 only blocks binding of vasopressin to the weaker site (5). In order to study the proximity of Tyr-49 to the peptide site, we have studied the effects of two spin-labeled peptides, previously shown to bind to the peptide site (7), on the longitudinal relaxation time, $T_1$, of the ortho ring protons (ortho to the hydroxyl) of Tyr-49. The spin labels are (A), (glycyl-L-phenylalanyl-amido)-4-2,2,6,6-tetramethyl-piperidine-1-oxyl, and (B), S-acetamido-3-(2,2,5,5-tetramethyl pyrrolidine-1-oxyl)-L-cysteinyl-L-tyrosine amide (Fig. 1).

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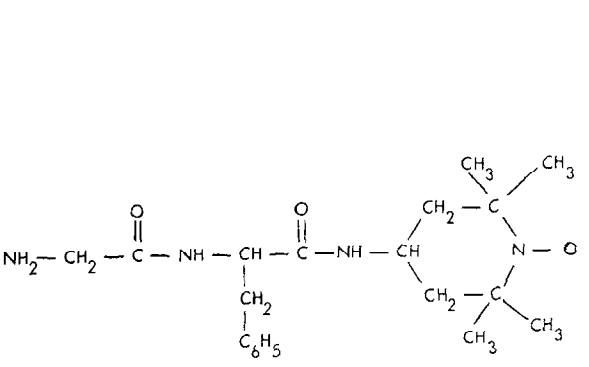
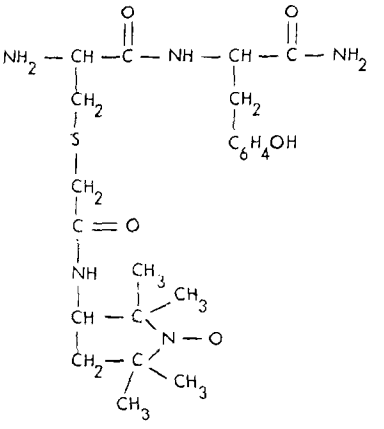


A



B

Figure 1 Left: (glycyl-L-phenylalanylamido)-4-2,2,6,6-tetramethyl-piperidine-1-oxyl (A); right: S-acetamido-3-(2,2,5,5-tetramethyl pyrrolidine-1-oxyl)-L-cysteinyl-L-tyrosine amide (B).

## MATERIALS AND METHODS

Bovine NP-I[1] and spin labels (A) and (B) were prepared as previously described (7,9). $NH_2$-TEMPO was obtained from Aldrich Chemical Co. and converted to its succinylated derivative, S-TEMPO, by reaction with succinic anhydride. All diamagnetic peptides were those previously described (9). Binding constants for spin labels and other peptides were those previously reported (7,9). Samples were deuterated prior to NMR studies by repeated lyophilization from $D_2O$. For NMR studies, NP-I was 25-50 mg/ml in approximately 0.1 M NaCl, pH 6.2 (pH meter reading in $D_2O$) containing $10^{-3}$ M EDTA; the temperature was 23 ± 1° C. Total spin label concentrations were verified by ESR under non-binding conditions (7) using $NH_2$-TEMPO as the standard. Fourier transform NMR studies were performed principally using the 220 MHz Varian spectrometer at Rockefeller University; a limited series of studies were also carried out on the 220 MHz Varian spectrometer of the Middle Atlantic NMR Facility. Peak positions for the ortho ring protons of Tyr-49 in the absence or presence of peptides were those previously reported (8). $T_2$ values were determined from line widths. $T_1$ values were determined by the inversion-recovery method (10) on the Rockefeller instrument and by the progressive saturation method (11) on the Middle Atlantic spectrometer. Data were fit using computer programs supplied by Nicolet. $T_1$ measurements on diamagnetic peptides in the presence of (B) indicated that the rapidly relaxing tyrosine protons of (B), at the concentrations used, did not generate artifacts in the measured $T_1$ of diamagnetic tyrosine protons (c.f., data with L-seryl-L-tyrosine amide in Results).

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[1] Abbreviations used are: NP, neurophysin; $NH_2$-TEMPO, 4-amino-2,2,6,6-tetramethyl piperidine-1-oxyl; S-TEMPO, (succinylamido)-4-2,2,6,6-tetramethyl-piperidine-1-oxyl; NMR, nuclear magnetic resonance; S-$CH_3$-CFI, S-methyl-L-cysteinyl-L-phenylalanyl-L-isoleucine amide; ESR, electron spin resonance.

## RESULTS

Effect of diamagnetic peptides on Tyr-49: In order to interpret the relaxation effects of spin labels, the effects of binding comparable diamagnetic ligands must be determined (12). In agreement with earlier studies (8), we calculated $T_2$ for the ortho ring protons of Tyr-49 in the absence of ligand as 0.014 sec. In the presence of saturating concentrations of the binding peptide S-$CH_3$-CFI, $T_2$ decreased to 0.013 sec; this result differs from earlier data (8) which may reflect the higher protein concentration used here. No $T_1$ values for the protons of Tyr-49 have been reported. From a series of 11 studies, we calculate the $T_1$ of the ortho ring protons to be 0.80 ± 0.05 (standard deviation) sec. In the presence of 9 mM S-$CH_3$-CFI (sufficient to give 90% saturation of the peptide site), $T_1$ was 0.75 ± 0.05 sec. The $T_1/T_2$ ratio can be shown (12) to indicate a rotational correlation time for Tyr-49 of $>10^{-8}$ sec both in the free protein and in the complex. Additionally, the high $T_1$ value, when considered together with the known kinetic constants (13) for NP-vasopressin interaction and the fact (8) that small peptides exchange much more rapidly with their NP complexes than does vasopressin, indicates that NP-I interaction with small peptides meets the fast exchange criteria (12) necessary for spin label distance measurements from $T_1$ data.

Effects of spin-labeled peptides and non-binding nitroxides in the absence and presence of diamagnetic peptides: Spin labels can potentially decrease the $T_1$ value of a given protein proton both when bound to the same protein molecule (intramolecular relaxation) and when free or bound to a different protein molecule (intermolecular relaxation) (12). Only intramolecular effects are relevant to distance calculations; so other effects must be subtracted. Studies in the absence of protein indicated that S-TEMPO (which doesn't bind to NP-I) and (A) and (B) were quantitatively similar in their relaxation effects on the ring protons of the peptide L-seryl-L-tyrosine amide. Additionally, (A) and (B) bound to NP-I were shown not to relax the C-2 proton of the single NP-I histidine any more effectively than comparable concentrations of free S-TEMPO.

Table I

Effects of S-$CH_3$-Cys-Phe-Ile $NH_2$ on the Relaxation Rate of the Ortho Ring Protons of Tyr-49

| Conditions | Observed[a] $T_1$(sec) | Observed[a] $1/T_1$ ($sec^{-1}$) | Calculated: Effect of S-$CH_3$-CFI $\Delta$ $1/T_1$ ($sec^{-1}$)[b] | Calculated: Paramagnetic effects of bound spin label $\Delta$ $1/T_1$ ($sec^{-1}$) |
|---|---|---|---|---|
| NP-I (2.5-5 mM) | 0.80 | 1.25 | | |
| NP-I (2.5-5 mM) + 9 mM S-$CH_3$-CFI | 0.75 | 1.33 | +0.08 | |
| NP-I (5 mM) + 1 mM S-TEMPO | 0.71 | 1.41 | | |
| NP-I (5 mM) + 1 mM S-TEMPO + 9 mM S-$CH_3$-CFI | 0.65 | 1.54 | +0.13 | |
| NP-I (4 mM) + 1 mM (A) | 0.64 | 1.56 | | +0.14[c] |
| NP-I (4 mM) + 1 mM (A) + 9 mM S-$CH_3$-CFI | 0.44 | 2.27 | +0.71 | +0.73[d] |
| NP-I (3 mM) + 0.4 mM (B) | 0.40 | 2.50 | | +1.18[c] |
| NP-I (3 mM) + 0.4 mM (B) + 9 mM S-$CH_3$-CFI | 0.43[e] | 2.32 | -0.18 | +0.93[d] |

[a] All values, except as noted, represent the average of two or more determinations. The standard deviation of the average $T_1$ in each series was equal to or less than 6% of the absolute value of the average.

[b] Calculated for each nitroxide as the difference between $1/T_1$ in the absence and in the presence of S-$CH_3$-CFI.

[c] Calculated as $1/T_1$ in the presence of spin label-$1/T_1$ in the presence of equivalent concentrations of S-TEMPO-0.08x(fractional saturation of the strong site). The last term corrects for the diamagnetic effects of binding. The calculation assumes, as indicated in the text, that both bound and free spin label have equivalent intermolecular relaxation effects. The possibility exists that bound spin label may be less effective than free spin label in intermolecular relaxation but can be shown not to have qualitative implications for the conclusions reached.

[d] Calculated as $1/T_1$ in the presence of spin label + 9 mM S-$CH_3$-CFI-$1/T_1$ in the presence of equivalent total concentrations of S-TEMPO + 9 mM S-$CH_3$-CFI.

[e] Single determination.

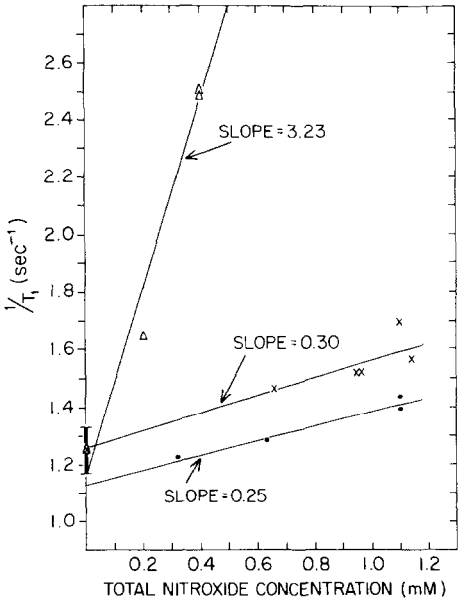


Figure 2 Effect of nitroxides on the relaxation rate, $1/T_1$, of the ortho ring protons of Tyr-49. •, S-TEMPO; X, Spin label (A); Δ, Spin label (B). Solid lines through the data points are the least squares fit. The vertical bar on the ordinate represents one standard deviation on either side of the average for results in the absence of nitroxide; the fact that the line representing the S-TEMPO data intersects slightly below the bar is not considered statistically significant.

Accordingly, we assume that the intermolecular effects of free and bound spin label are equivalent and judge the magnitude of these effects from the effects of S-TEMPO on Tyr-49 (see also Footnote c, Table I).

Fig. 2 shows the effects of increasing concentrations of (A), (B) and S-TEMPO on the relaxation rate ($1/T_1$) of the ortho protons of Tyr-49. From known binding constants (7), the protein is 10-15% saturated with (A) and (B) at the highest concentrations shown. However, the similarity of the slopes obtained with (A) and S-TEMPO indicate very little extra relaxation resulting from binding of (A). Significant extra relaxation results from the binding of (B), suggesting that its nitroxide is closer to Tyr-49 in the complex than the nitroxide of (A).

The diamagnetic peptide S-$CH_3$-CFI, like other binding peptides (7,9), competes with (A) and (B) for the peptide-binding site of NP-I and would therefore be expected to reduce any extra relaxation observed with (A) and (B) relative to S-TEMPO. However, as shown in Table I, addition of sufficient S-$CH_3$-CFI to cause virtually complete spin label displacement had almost no effect on the extra relaxation produced by (B) and markedly increased the relaxation effects of (A); only trivial effects of S-$CH_3$-CFI, attributable largely to the diamagnetic effects of binding, were seen on relaxation with S-TEMPO. The above studies were performed using the Rockefeller spectrometer; the particularly unexpected effect of S-$CH_3$-CFI on relaxation by (A) was qualitatively confirmed in a limited study with (A) at the Middle Atlantic NMR facility using $NH_2$-TEMPO as the non-binding reference. The effects of S-$CH_3$-CFI on relaxation by (A) and (B), when considered together with its lack of effect on relaxation by non-binding nitroxides are inconsistent with a single site binding model for (A) and (B). Alternatively these results can be explained by a 2-binding site model in which the weaker site lies closer to Tyr-49 than the strong site and has increased occupancy by spin label in the presence of S-$CH_3$-CFI.

The Two-Binding Site Model: In accord with thermodynamic studies of peptide-binding (5-7), the model assumes that the weaker site has no more than 1/100 the affinity of the stronger site. The affinities of different peptides, relative to each other, are assumed to be the same at both sites (although this need not be the case) but, because the free concentration of S-$CH_3$-CFI used here is low relative to its assumed binding constant to the weaker site ($<50\ M^{-1}$), it cannot significantly displace (A) or (B) from the weaker site. There are two mechanisms by which occupancy of the strong site by S-$CH_3$-CFI can potentially increase occupancy of the weak site by spin label; these can be distinguished by results obtained with (A). In the absence of S-$CH_3$-CFI, at the protein concentrations used, binding constants (7) indicate that 50% of the total (A) is bound to the strong site of NP-I. Displacement of (A) from the strong site

therefore doubles the concentration of free (A). If the two sites are independent, this will maximally double the weak site occupancy and therefore maximally double the paramagnetic contribution to $1/T_1$. However, if binding to the weaker site is cooperatively linked to occupancy of the stronger site, then the fact that total strong site occupancy increases from 0.15 in the presence of (A) alone (vide supra) to >0.9 when S-$CH_3$-CFI is added (vide supra) can lead to a 6-fold increase in weak site occupancy independently of an increase in free (A). The paramagnetic contribution of bound (A) to the relaxation rate increases significantly more than 2-fold when S-$CH_3$-CFI is added (Table I) suggesting cooperative interactions between the strong and weak sites. The slightly decreased paramagnetic contribution of bound (B) in the presence of S-$CH_3$-CFI (Table I) presumably arises from loss of relaxation from (B) at the strong site, largely compensated for by increased relaxation from (B) at the weak site.

## DISCUSSION

The presence of a secondary binding site for small peptides, in close proximity to Tyr-49, is in remarkable agreement with the observation (5) that nitration of Tyr-49 prevents binding of vasopressin to the second hormone-binding site and suggests that this effect is steric. Preliminary calculations of the distances between Tyr-49 and nitroxides at the weak site, using the above model and a correlation time of $10^{-8}$ sec, place the unpaired electrons of both (A) and (B) less than 5 Å from Tyr-49. Additionally, the present studies extend the two-site model in that they suggest positive cooperative interactions between the sites. However, an alternate model for the site-site interactions cannot be strictly excluded. Thus it is possible that these interactions arise from two relatively equivalent sites, both somewhat distant from Tyr-49, occupancy of one of which weakens the other and displaces it towards Tyr-49.

The question as to the proximity of Tyr-49 to the strong site remains of interest. Preliminary calculations suggest that a large fraction of the enhanced relaxation by (B) in the absence of S-$CH_3$-CFI arises from the strong site. It therefore remains possible that the strong site is close enough to

Tyr-49 to account for the observed Nuclear Overhauser data, but the present results suggest that some of these effects may have been mediated by the presence of peptide at the weak site.

REFERENCES

1. Nicolas, P., Camier, M., Dessen, P. and Cohen, P., J. Biol. Chem., 251, 3965-3971 (1976).
2. Glasel, J.A., McKelvy, J.F., Hruby, V.J. and Spatola, A.F., J. Biol. Chem., 251, 2929-2937 (1976).
3. Breslow, E., Ann. N.Y. Acad. Sci., 248, 423-441 (1975).
4. Capra, J.D., Kotelchuck, D., Walter, R. and Breslow, E., Proc. Natl. Acad. Sci., U.S.A., 69, 431-434 (1972).
5. Cohen, P., Camier, M., Wolff, J., Alazard, R., Cohen, J.S. and Griffin, J.H., Ann. N.Y. Acad. Sci., 248, 463-479 (1975).
6. Chaiken, I.M., Randolph, R.E. and Taylor, Hope C., Ann. N.Y. Acad. Sci., 248, 442-450 (1975).
7. Lundt, S.L. and Breslow, E., J. Phys. Chem., 80, 1123-1126 (1976).
8. Balaram, P., Bothner-By, A.A. and Breslow, E., Biochemistry, 12, 4695-4704 (1973).
9. Breslow, E., Weis, J. and Menendez-Botet, C.J., Biochemistry, 12, 4644-4653 (1973).
10. Vold, R.L., Waugh, J.S., Klein, M.P. and Phelps, D.E., J. Chem. Phys. 48, 3831-3832 (1968).
11. Freeman, R. and Hill, H.D.W., J. Chem. Phys., 54, 3367-3377 (1971).
12. Krugh, T.R. in "Spin Labeling Theory and Applications" (L.J. Berliner, ed.), Pp. 339-370, Academic Press (1976).
13. Pearlmutter, A.F. and McMains, C., Biochemistry, 16, 628-633 (1977).