Detection of Thymosin $\beta_4$ in Situ in a Guinea Pig Cerebral Cortex Preparation Using $^1$H NMR Spectroscopy*

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In the present work we have investigated the macromolecules that contribute to the brain $^1$H NMR spectrum. The cerebral cortex showed distinct resonances at the uncrowded methyl- and methylene-chemical shift scale of the spin-echo $^1$H NMR spectrum. The peaks at 1.22 and 1.40 ppm (relative to the methyl protons of N-acetyl aspartate at 2.02 ppm) arise from cerebral macromolecules without evidence for co-resonances from low molecular weight metabolites as shown by the spin-spin relaxation decays of these resonances. In addition to these NMR signals, peaks at 0.9 and 1.7 ppm from macromolecules were detected. These resonances are from proteins, and we have identified the polypeptides that contributed to the $^1$H NMR peaks. Two proteins that were present at concentrations of 250 and 350 $\mu$g/g of dried tissue showed $^1$H NMR spectra that resembled the macromolecular pattern in the cerebral $^1$H NMR spectrum. They were identified as thymosin $\beta_4$ and histone H1, respectively. Thymosin $\beta_4$ was present in soluble high speed cytoplasmic fraction and in P2 pellet, whereas histone H1 was detected in nuclear enriched fraction. A chemical shift-correlated two-dimensional $^1$H NMR spectrum of thymosin $\beta_4$ in vitro revealed a coupling pattern that matches the macromolecule in the cerebral cortex which we have previously noted (Kauppinen R. A., Kokko, H., and Williams, S. R. (1992) J. Neurochem. 58, 967–974). On the basis of both one- and two-dimensional NMR evidence, subcellular distribution and high concentration, we assign the $^1$H NMR signals at 0.9, 1.22, 1.40, and 1.7 ppm in the cerebral cortex to thymosin $\beta_4$.

$^1$H NMR spectroscopy provides a nondestructive assessment and determination of a number of cytosolic metabolites in the intact brain including lactate, NAA, glutamate, glutamine, $\gamma$-aminobutyric acid, creatine, choline-containing compounds, inositol, and taurine (1). The contribution of macromolecules of either extracranial or intracranial origin to the brain $^1$H NMR spectrum is well appreciated, and therefore special methods are required to filter out these signals to monitor cerebral metabolites (2, 3). For instance, a broad "hump" arising from subcutaneous fat has to be subtracted before observation at CH$_3$ of lactate or alanine at 1.33 and 1.47 ppm, respectively, when $^1$H NMR is recorded with surface coils (2, 4, 5). So-called spectral editing is often used for this purpose (2, 6).

We have shown previously another type of contribution of macromolecules to the $^1$H NMR spectrum recorded from a superfused cerebral brain slice preparation (7). Our data showed distinct peaks at the methyl- and methylene region of the chemical shift scale of the $^1$H NMR spectrum at 400 MHz. The resonances at 0.88, 0.94, 1.22, and 1.40 ppm of the cerebral preparation had shorter T$_2$ relaxation times and lower saturation factors than lactate or NAA, indicating that the peaks have significantly different relaxation properties than the cytoplasmic metabolites (7). Demonstration of scalar coupling between the peaks from 0.8 to 1.4 ppm and the resonances down-field in the methine site by using inhibition of phase modulation as an indicator confirmed that the peaks mentioned above did not have a significant contribution from magnetically equivalent CH$_3$ groups of endogenous lipids of brain (7). The compounds were most likely proteins that are sufficiently mobile in the intact cerebral cortex, and they were soluble in 5% (w/v) perchloric acid (7). In the present paper we have investigated cerebral acid-soluble polypeptides and proteins and studied their one-dimensional and two-dimensional $^1$H NMR. Our results show that the distinct peaks in the spin-echo $^1$H NMR spectrum are assigned to thymosin $\beta_4$ with negligible contamination from other cerebral proteins.

EXPERIMENTAL PROCEDURES

Preparation of Cerebral Brain Slices, Incubations, and $^1$H NMR of the Preparation—The cerebral slices from Dunkin-Hartley strain male guinea pigs (250-400 g) were prepared and incubated in Krebs-Henseleit buffer containing 124 mM NaCl, 5 mM KCl, 1.2 mM MgSO$_4$, 1.2 mM CaCl$_2$, 26 mM NaHCO$_3$, 1.2 mM KH$_2$PO$_4$, and 10 mM d-glucose equilibrated with O$_2$/CO$_2$ (85%/15%) for 30-45 min at 37°C as described previously (8, 9). Slices were superfused in an apparatus described previously (8). $^1$H NMR spectra were recorded using a Bruker 15-mm $^1$H NMR probe in a 9.4 tesla vertical magnet interfaced with a Bruker AM-400 spectrometer. $^1$H NMR spectra (spectral width of 5 kHz, 4,096 data points, interpulse interval of 1.7 s) were recorded using a spin-echo sequence incorporating solvent suppression pulses as described previously (2, 10). Chemical shifts are expressed relative to a value of 2.02 ppm for the methyl resonance of NAA. Spin-spin relaxation decay constants were determined using a spin-echo sequence as described previously (7, 9).

Extraction of Brain Tissues—Brain cortices (1 volume) from killed animals were homogenized into 0.2, 0.9, 1.2, or 2.0 M ice-cold perchloric acid in 145 mM NaCl, 9 mM KCl (3 volumes) using a Potter-Elvehjem homogeniser. After a 30-min incubation on ice, homogenates were centrifuged at 14,500 × g for 30 min. Clear supernatants were neutralized with 3.5 M K$_2$CO$_3$ to pH 6.5 and freeze-dried before further
analyses. Protein concentration was determined using the method of Bradford (11), with bovine serum albumin as standard. A high-speed-soluble cytosolic fraction of cerebral cortex was obtained according to the method of Park et al. (12), P2 pellet according to Nicholls (15), and a nuclear-enriched fraction according to McEwen et al. (14). Fractions were extracted with 0.2 M perchloric acid and neutralized to pH 6.5 before further analyses.

Purification and Sequencing of Proteins—Neutralized and freeze-dried cerebral extracts were dissolved in 0.1% trifluoroacetic acid in H2O and injected into a C18/C6 reversed-phase fast protein liquid chromatography column (ProRPC HR 5/10, Pharmacia LKB Bio-technology Inc.). Elution was performed using a stepwise gradient of 0.1% (w/v) trifluoroacetic acid in H2O to 0.1% (w/v) trifluoroacetic acid in 70% (v/v) acetonitrile over 100 min. Some of the protein fractions obtained by a C18/C6 reversed-phase column were purified further using a C8/C18 reversed-phase column (PepRPC HR 5/10, Pharmacia) with an elution procedure similar to that in the C18/C6 chromatography.

Proteins in acid extracts were quantified using a Vydac C4 reversed-phase column (250 × 4.6 mm, 5-mm particle size, 350 A pore size) in a Hewlett-Packard 1090 liquid chromatograph with a Varian 4270 integrator as described previously (15). Purified HM01 thymus (16), histone H1 from rat and calf thymus (17), and bovine ubiquitin (Sigma) were used as standards in the HPLC analysis. The high density SDS-polyacrylamide gel electrophoresis and isoelectric focusing were carried out using a Pharmacia (Pharmacia). Gels were stained with silver nitrate.

Amino acid sequences of some of the proteins were determined using an Applied Biosystems 477A Sequencer with a 120A analyzer. The proteins purified as described above were digested with trypsin (sequencing grade, Sigma) in 6 M urea, 0.5 M NH4HCO3 at pH 8.0 for 12 h at 37 °C. The peptides were separated using the C8/C18 column (PepRPC HR 6/10) as described above. Amino-terminal sequence of thymosin β4 was verified by deblocking using the method of Wellner et al. (18) and thereafter exposing the peptide to Edman degradation in the automatic Sequencer.

NMR Analysis of Extracts and Purified Proteins—1H NMR spectra of proteins in 100 mM NaCl, 4 mM KH2PO4 in D2O (pH 6.5) were acquired using 180° pulses repeated every 3.1 s (spectral width, 5 kHz; 16,384 data points) and using low power presaturation at the residual HOD signal. Chemical shifts of peaks are referenced to (trimethylsilyl)propionic acid at 0 ppm, and (trimethylsilyl)propionic acid was also used as a concentration standard. Two-dimensional COSY spectra were acquired using either a sequence by Nagayama and Wütrich (19) or a sequence described by Aye et al. (20), with presaturation of the HOD resonance during the relaxation delay. In the first case, the pulse sequence was modified so that the excitation was obtained by a semiselective 1-3-3-pulse with maximal excitation adjusted to 1.33 ppm. The two-dimensional data matrices in solution study were either 512 × 1,024 or 1,024 × 2,048 data points for F2 and F1 dimensions, respectively.

Reagents—Reagents for chromatographic analyses were from Pharmacia or Bio-Rad. Ubiquitin was purchased from Sigma. All other standard chemicals were of analytical grade and were obtained either from Sigma, Boehringer Mannheim, or Merck.

RESULTS

1H NMR of the Cerebral Preparation—A 1H NMR spectrum (Fig. 1A) recorded from the cerebral preparation using presaturation as a means of water suppression shows huge peaks at 0.7 and 1.1 ppm, respectively, which are much more intense than the broad signals at the chemical shift of the CH2 of NAA (at 2.02 ppm) and CH2 of lactate (1.33 ppm). Fig. 1B shows a 1H NMR spectrum from the same preparation acquired using a spin-echo sequence with an echo time of 20 ms. It is evident that there was a well resolved, sharp peak at 2.02 ppm and a number of broader signals between 0.7 and 1.7 ppm that at 2.02 ppm. This spectrum shows that there was a strong contribution from macromolecules in Fig. 1A and that there are a number of 1H NMR signals with very short T2 (<10 ms) which were filtered out in a spin-echo experiment. In the present study the origin of macromolecular signals present after a modest T2 filtering (Fig. 1B) was investigated.

In Fig. 1C, the signal intensities of peaks at 1.22 and 1.40 ppm are plotted against the spin-echo delay. Intensities of both peaks decayed according to single exponential fits with decay constants of 56 and 60 ms, respectively. These T2 values are shorter than what we have determined previously for NAA (93 ms) and lactate (84 ms) (7). The decay pattern of peaks at 1.22 and 1.40 ppm indicates that they arise from macromolecules with homogeneous relaxation times but T2 values distinct from metabolites.

Identification of 1H NMR-detectable Cerebral Macromolecules—Our previous data indicated that the cerebral macromolecules that gave one-dimensional 1H NMR spectra in the region from 0.8 to 1.7 ppm similar to the cerebral preparation in Fig. 1B are acid-soluble (7), which would ease their identification. A reversed-phase chromatogram of cerebral cortex extracted with 0.9 M perchloric acid shown in Fig. 2A contains nine major fractions. SDS-gel electrophoresis shows that fraction 2 probably consists of very small peptides, whereas fractions 3-5 revealed several polypeptide bands ranging from 1.5 to 17 kDa and a 45-kDa protein in fraction 4 (Fig. 2B). Fig. 3 shows that only fractions 4 and 5 from the reversed-phase chromatography produced one-dimensional 1H NMR spectra that were compatible with the pattern of the unidentified cerebral macromolecule in Fig. 1B.

SDS-gel electrophoresis (Fig. 2B, lane 4) suggests that fraction 4 consisted of low molecular mass, mainly 7-kDa polypeptides. Fraction 4 was further resolved into two polypeptides using a more hydrophobic C6/C18 reversed-phase column (Fig. 4A). Both fractions showed one-dimensional 1H NMR spectra similar to that in Fig. 3 (FR 4; not shown). The fractions did not yield amino acid sequences as analyzed using straight Edman degradation. The polypeptide from the large fraction was digested with trypsin and the major tryptic peptide (Trp-12) purified with the C6/C18 column, sequenced. The amino acid sequence of this peptide (Fig. 4B) was identical to the bovine thymosin β4 (21). The amino-terminal sequence of this polypeptide was confirmed from residues 1-22 after deblocking with trifluoroacetic acid (Fig. 4B). The
Cerebral Thymosin \( \beta_4 \) by \(^1\)H NMR

**Fig. 2.** Reversed-phase chromatogram (A) of a perchloric acid extract from cerebral cortex, and SDS-gel electrophoresis (B) from the corresponding fractions. Cerebral cortex was extracted with 0.9 M perchloric acid, and ProRPC analysis was carried out as described under "Experimental Procedures." One mg of protein was injected into the column. \( A_214 \) is indicated relative to 0.1% trifluoroacetic acid in water. The lanes in B are numbered according to the fractions in A. Two to four \( \mu \)g of protein was loaded in each lane. a and b indicate molecular mass standards in kDa.

**Fig. 3.** \(^1\)H NMR spectra of protein fractions (FR) from the reversed-phase chromatography of cerebral perchloric acid extracts. Fractions from ProRPC chromatograms were freeze-dried, pooled (0.4–0.6 mg of protein), and dissolved in 4 mM KH\(_2\)PO\(_4\), 100 mM NaCl in D\(_2\)O, pH 6.5 and \(^1\)H NMR acquired as described under "Experimental Procedures." Each spectrum is a sum of 200 scans. An exponential function corresponding to line broadening of 2 Hz was applied prior to Fourier transformation.

Small protein peak preceding thymosin \( \beta_4 \) was not sequenced, but we tentatively assigned it to thymosin \( \beta_{10} \) according to the retention time in a similar analysis and its concentration relative to thymosin \( \beta_4 \) (22).

Fraction 5 (Fig. 2A), which contained acid-soluble proteins with same type of one-dimensional \(^1\)H NMR spectrum as thymosin \( \beta_4 \), was a mixture of polypeptides with molecular masses ranging from 13 to 18 kDa and a protein of 42 kDa (Fig. 3B, lane 5; see below for their identification). Fractions 6 and 7 contained mainly ubiquitin as identified by the retention time using bovine ubiquitin as a standard, but also another polypeptide in fraction 6 with a molecular mass of 18 kDa (Fig. 3B, lane 6).

The results above suggest that thymosin \( \beta_4 \) is the most abundant cerebral polypeptide that has a one-dimensional \(^1\)H NMR spectrum compatible with the macromolecular pattern in the spin-echo experiment of the slice preparation (Fig. 1B). It may be that the relative solubility of individual polypeptides differed as a function of perchloric acid concentration. We therefore quantified acid-soluble proteins from the cerebral cortex extracted with 0.2, 0.9, 1.2, or 2.0 M perchloric acid. Typical HPLC chromatograms from 0.2 and 0.9 M perchloric acid extracts are shown in Fig. 5, A and B.

\(^1\)H NMR analysis showed that there are four fractions in the HPLC chromatogram (fractions 1–3 and 5) which have to be identified as their spectra resembled that shown in Fig. 3 (FR 4) and the fraction 4 as it is present at high concentration. The proteins were as follows: 1, HMG 17, 2, thymosin \( \beta_4 \); 3, prothymosin \( \alpha \); 4, ubiquitin; and 5, histone H1. These proteins were identified using (i) purified proteins as standards and by determining their mobility in an SDS-gel electrophoresis (ubiquitin, histone H1); (ii) according to both molecular mass determined by SDS-polyacrylamide gel electrophoresis (11 kDa) and to isoelectric focusing (pI 4.2, prothymosin \( \alpha \); Ref. 15); or (iii) by determining peptide sequences (HMG 17 and thymosin \( \beta_4 \)). Retention times of the assigned proteins were 6.80 ± 0.21, 17.60 ± 0.13, 20.83 ± 0.19, 38.68 ± 0.20, and 41.5 ± 0.15 min, respectively.

The concentrations of these proteins are given in Table I. It ought to be noted that some 2.5-fold quantity of protein was soluble in 0.2 M perchloric acid compared with other concentrations studied. Solubilities of ubiquitin and histone H1 decreased as the concentration of acid was elevated from 0.2 to 0.9 M or above, whereas concentrations of HMG 17, thymosin \( \beta_4 \), or prothymosin \( \alpha \) were similar at each perchloric acid concentration (Table I). All perchloric acid concentrations studied were as effective in extracting low molecular weight metabolites NAA and (phospho)creatine (Table I).

One-dimensional \(^1\)H NMR spectrum of histone H1 showed similarities to that of thymosin \( \beta_4 \) (Fig. 6, B versus A), but the ratios of peaks of 1.22, 0.94, and 0.88 ppm to 1.4 ppm
Two proteins were in the fraction and their concentrations were determined using ubiquitin as a standard. Assumptions are assigned as follows. NMR as described under "Experimental Procedures." Values (means + S.E.) in mg of protein or metabolite/g of extract dry weight are representative for three or four independent experiments. "(1)" after histone H1 indicates that it was present only in one of three experiments at given perchloric acid concentrations. ND, not detectable.

Table 1

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<tr>
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<td>441 ± 50</td>
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<td>511 ± 72</td>
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FIG. 5. HPLC chromatograms of perchloric acid–extracted cerebral cortex. Cerebral corticies were extracted with 0.2 M (A) and 0.9 M perchloric acid (B), and C, reversed-phase HPLC analyses were performed as described under "Experimental Procedures." Fractions are assigned as follows. 1, HMG 17; 2, thymosin β4; 3, prothymosin α; 4, ubiquitin; and 5, histone H1. 1.25 mg (A) and 0.63 mg (B) of protein was injected into the column.

Table 2

Protein, (phospho)creatine, and NAA concentrations in the perchloric acid extracts from cerebral cortex

Cerebral corticies were extracted into perchloric acid in 145 NaCl, 9 mM KC1 at the concentrations required. Protein was assayed using a calorimetric method. Proteins in the extracts were separated by an HPLC method as described under "Experimental Procedures," and their concentrations were determined using ubiquitin as a standard. (Phospho)creatine ((P)creatine) and NAA were quantified using 1H NMR as described under "Experimental Procedures." Values (means ± S.E.) in mg of protein or metabolite/g of extract dry weight are representative for three or four independent experiments. "(1)" after histone H1 indicates that it was present only in one of three experiments at given perchloric acid concentrations. ND, not detectable.

Table 3

Perchloric acid concentration

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were significantly smaller in H1 than in thymosin β4. 1H NMR spectra of HMG 17 and prothymosin α very much resembled that of H1 (not shown). It is likely that the latter two proteins were in the fraction 5 of the ProRPC chromatogram (Fig. 2, A and B). None of these proteins eluting between 27 and 35 min in the chromatogram of 0.2 M perchloric acid extract had an 1H NMR spectrum similar to that of thymosin β4 (not shown). Therefore, they were not identified further.

The HPLC chromatogram of acid-extracted soluble cytosol of cerebral cortex contained thymosin β4 and ubiquitin at a ratio of 0.38; but HMG 17, prothymosin α, and histone H1 were not detectable (Fig. 7A). The P2 pellet contained also thymosin β4 and ubiquitin (a concentration ratio of 0.19) and also a small amount of histone H1 (Fig. 7B). HMG 17, prothymosin α, and histone H1 were present in the nuclear enriched pellet in substantially greater ratios relative to ubiquitin (Fig. 7C) than in either in the soluble cytosol or in the P2 pellet.

Two-dimensional NMR of Purified Thymosin β4 and Histone H1—It is necessary for an unambiguous identification to detect the scalar couplings in the candidate polypeptide compatible with those we have reported previously for the macromolecule in situ (7). A two-dimensional COSY contour plot (Fig. 6C) shows that there are the following scalar correlations within the polypeptide: between 1.22 and 4.20 ppm, between 1.72 and 3.0 ppm, between 1.91 and 0.94 ppm, between 1.67 and 0.94 ppm, between 1.42 and 0.88 ppm, and several short range correlations at regions of 2.3–2.5 ppm. In a COSY spectrum of thymosin β4 acquired using the sequence by Aue et al. (20) correlations also between 1.40 and 3.1 ppm, between 1.33 and 4.11 ppm were observable (not shown). These correlations could not be detected in Fig. 6C, as the resonances close to water (4.7 ppm) received little excitation by the 1-3-3-1 pulses. Of the correlations in thymosin β4 molecule, couplings between 1.22 and 4.2, 1.40 and 4.3, 1.9 and 0.94, 1.67 and 0.94, and 1.67 and 0.88 ppm are compatible with those we have reported previously (indicated by arrows in Fig. 6C) using the inhibition of phase modulation as an indicator of scalar coupling between the macromolecular resonances in situ (7). The cross-peak at 1.7 and 3.0 ppm was also present in histone H1, which did not have any other cross-peaks in a COSY contour plot acquired with the same pulse sequence as that of thymosin β4 in Fig. 6C (not shown).

Discussion

The present results demonstrate that even when T2 filtering is used, significant contributions from polypeptides are present in the 1H NMR spectrum of the cerebral cortex. The analysis of cerebral extracts shows that peaks at 0.9, 1.22, and 1.40 ppm of the 1H NMR spectrum could arise from two individual proteins, i.e., from cytoplasmic thymosin β4 and from nuclear histone H1. Our data favor the conclusion that thymosin β4 is the polypeptide that gives rise to the peaks at 0.88, 0.94, and 1.22 ppm, and it is the predominant contributor to that at 1.40 ppm, as supported by both NMR and biochemical facts.

By comparing a 1H NMR spectrum of cerebral preparation (Fig. 1B) with the one-dimensional spectra of purified thymosin β4 and histone H1 (Fig. 6, A and B), it is evident that the ratios of peaks at 0.9 (arising from valine, leucine, and isoleucine) and 1.22 (from threonine) ppm to 1.40 (from alanine) ppm are closer to each other in thymosin β4 than in the histone. The scalar couplings of peaks in thymosin β4 at the region from 0.8 to 2.0 ppm are compatible with the one we have determined for the in situ macromolecule(s) (7). We were able to detect only a cross-peak of 1.7–3.0 ppm in the histone, however, with the COSY method or by using the inhibition of phase modulation. The concentrations and sub-

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**Fig. 6.** $^1$H NMR spectra of purified thymosin β4 (A) and histone H1 (B) and a COSY spectrum of thymosin β4 (C). Conditions as in Fig. 3. A and B, are sums of 50 scans. An exponential function corresponding to line broadening of 2 Hz was applied before Fourier transformation. C, two-dimensional COSY data matrices were 1,024 × 2,048; digital resolution was 2.2 Hz both in F1 and F2 dimensions. In A and C, 1.8 mg/ml and in B, 5 mg/ml protein was dissolved in 4 mM KH$_2$PO$_4$, 100 mM NaCl in D$_2$O, pH 6.5.

**Fig. 7.** HPLC chromatograms of perchloric acid-extracted soluble cytosolic fraction (A), P2 pellet (B), and nuclear enriched fraction (C). Subcellular fractions were obtained, extracted, and analyzed by HPLC as described under "Experimental Procedures." 17.7 μg (A), 19.6 μg (B), and 19.4 μg (C) of protein was injected into the column. Peaks are assigned as in Fig. 5.

Cellular distributions of other proteins with a type of $^1$H NMR spectrum similar to that of thymosin β4, i.e. HMG 17 and prothymosin α (Table I) are so low that their contributions to the signals from 0.8 to 1.4 ppm of the cerebral $^1$H NMR spectrum must be negligible. We show that thymosin β4 is present in the soluble cerebral cytosolic and P2 fractions (which contains nerve endings and myelin among others), whereas histones are not detectable in these fractions. It has been shown previously that thymic prothymosin α and parathymosin are distributed between nuclear and cytosolic compartments, whereas thymosin β4 is present only in the cytosol (23). Our results support similar subcellular distributions of thymosin β4 and prothymosin α also in the cerebral cortex. The cytoplasmic localization strongly favors $^1$H NMR "visibility" of thymosin β4 over histones. This conclusion of NMR detectability of proteins has been suggested previously (3, 24). In fact, recent evidence suggests that sequestration of some metabolites, for instance brain glutamate into noncytoplasmic compartments, may influence their detection by $^1$H NMR (8, 25). On the other hand, in the case of macromolecules there must be parts of the molecule sufficiently mobile or a given fraction of the total pool free before the "sharp" NMR resonances of the molecule are detectable.

Histone H1 has larger molecular mass (about 27 kDa) than thymosin β4 (about 5 kDa). The $T_2$ of signals at 1.22 and 1.40 ppm of these purified proteins in vitro are 136 and 204 ms for histone H1 and 211.9 and 187.6 ms for thymosin β4. This suggests that it is impossible to discriminate between these two proteins according to their $T_2$ if they both are "free." It is known that histone H1 is bound to DNA and links adjacent nucleosomes in nuclei (26). Using one-dimensional $^1$H NMR analysis Cary and co-workers (27) have shown that the basic regions of histone H6 which are rich in proline and arginine bind to DNA, and this will mask one-dimensional $^1$H NMR peaks at the α-CH region, i.e. at the chemical shift region of 3–4 ppm. The same type of interaction has been reported for HMG proteins which bind to nucleosomal core DNA interacting at the same site as core histones (26–28). $^1$H NMR of HMG 17 have revealed that peaks from proline (at 3.7 ppm) and from arginine (at 3.2 ppm) became "invisible" as the HMG protein interacts with DNA at physiological ionic strength (27, 29). The in vitro one-dimensional $^1$H NMR studies quoted above indicate that the parts of the histone H1 molecules that contains alanine, threonine, valine, leucine, and isoleucine may be mobile.

Previously, a COSY spectrum of dead rat brain acquired using the method by Aue et al. (20) revealed a correlation between 1.7 and 3.0 ppm which was separate from the cross-peak of γ-aminobutyric acid at 1.91–3.0 ppm (3). The same, strong correlation is present in a COSY spectrum recorded from the superfused, metabolically viable cerebral preparation. Because it is necessary for a spin system to retain the multiplet structure to give a correlation peak in a COSY experiment, the chemical groups within a molecule of origin must be free. The binding of thymosin β4 to cytoskeletal structures in vitro has been suggested (30). In thymosin β4, a
cross-peak of 1.7 and 3.0 ppm could arise from scalar coupling between $\delta$-CH$_2$ and e-CH$_2$ of lysine residues (31). It may be that the cross-peak in a COSY experiment (3) originates from the polypeptide, but this assignment must remain tentative.

Thymosin $\beta 4$ is a ubiquitous protein, but its function in the brain remains to be delineated. It has been shown that it exhibits the endocrine effect in the hypothalamus by inducing not only in the slice preparation, and $^1$H NMR may open a window for this study on mouse brain (34). It has been localized to oligodendrocytes using immunohistochemistry, and a role in myelination has been postulated (33). Its concentration in the cerebral cortex undergoes a developmental regulation decreasing some 20% during maturation, and $^1$H NMR may open a new, nondestructive possibility to seek a function for this polypeptide in the brain.

REFERENCES