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In Vitro Messenger Ribonucleic Acid Directed Synthesis and Processing of an Immunologically Identified Precursor to Tetradecapeptide Somatostatin from Bovine Hypothalamus†

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ABSTRACT: mRNA isolated from bovine hypothalami was used to direct the in vitro synthesis of a precursor to the tetradecapeptide somatostatin. When a rabbit reticulocyte lysate translation system supplemented with [^{35}S]cysteine was used, a protein of apparent molecular weight 15 500 was identified as preprosomatostatin by reaction with specific rabbit antibodies. Cotranslational addition of dog pancreas microsomal membranes yielded an unglycosylated pro form of 14 500 daltons, implying the removal of a short signal sequence.

The tetradecapeptide somatostatin (SRIF)[1] has been suggested to function not only as a hormone but also as a neurotransmitter and as a local regulatory agent (Schally, 1978; Guillemin, 1978; Cohn & Cohn, 1975). Although widely occurring in vertebrate tissues, it appears to be synthesized mainly in the hypothalamus and the endocrine pancreas, in a ribosome-dependent manner via a much larger precursor molecule (Ensinck et al., 1978; Noe et al., 1979; Joseph-Bravo et al., 1980; Patzelt et al., 1980). It thus conforms to the model already established for the biosynthesis of other peptide hor-

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; IgG, immunoglobulin G fraction; NpII, bovine neurophysin II; anti-NpII, rabbit antibodies to bovine neurophysin II; ppAVP/NpII and pAVP/NpII, prepro and pro forms, respectively, of the arginine vasopressin/neurophysin II common precursor; SRIF, tetradecapeptide somatostatin; anti-SRIF, rabbit antibodies to synthetic somatostatin; ppSRIF and pSRIF, prepro- and prosomatostatin, respectively; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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mones such as adrenocorticotropin (Roberts & Herbert, 1977; Nakanishi et al., 1979), the enkephalins (Stern et al., 1980), or arginine vasopressin (Russell et al., 1980; Schmale & Richter, 1980). Recently, in vitro translation of mRNA from tissues actively synthesizing the peptide hormone has confirmed the biosynthesis of somatostatin via a longer precursor (Shields, 1980; Joseph-Bravo et al., 1980). In the fish pancreas system multiple genes give rise to precursors of about 12 000–13 000 daltons (Hobart et al., 1980; Goodman et al., 1980a), whereas in the mouse a prepro form of about 15 000 daltons has been reported (Joseph-Bravo et al., 1980).

Because of the convenience of working with the fish pancreas system, much information is available on somatostatin biosynthesis in this tissue, including mRNA sequences (Hobart et al., 1980; Goodman et al., 1980a). However, it is not clear to what extent, if any, these data can be applied to the synthesis of mammalian somatostatin precursors, for which relatively little information is available. Here we show that in vitro translation of bovine hypothalamic mRNA yields a primary translation product of apparent molecular weight (M_r) 15 500, containing an amino acid sequence immunologically similar to synthetic somatostatin. On cotranslational addition of dog pancreas microsomal membranes, an unglycosylated pro form is synthesized which would appear to have lost a very short signal sequence.

Materials and Methods

Antisera to synthetic somatostatin (anti-SRIF) were obtained from Bioproducts, Brussels, and from Ferring GmbH, Kiel. The IgG fractions were prepared from these by precipitation with 40% saturated ammonium sulfate (Schmale & Richter, 1980). Synthetic somatostatin was from Peninsula, San Carlos, CA, or from Bioproducts, Brussels. Bovine mRNA was isolated by sodium dodecyl sulfate/phenol/chloroform extraction of hypothalamic polysomes, followed by oligo(dT)–cellulose chromatography as previously described (Richter et al., 1980). The mRNA from rat hypothalami, used for comparison, was isolated by the guanidinium thiocyanate procedure (Chirgwin et al., 1979) and oligo(dT)–cellulose chromatography. mRNA was translated in a rabbit reticulocyte lysate system (New England Nuclear) supplemented with [^{35}S]cysteine and/or [^{35}S]methionine. Where indicated, nuclease-treated dog pancreas microsomal membranes (0.05 OD_{280} unit per 25 μL of reaction mixture; Blobel & Dobberstein, 1975; Warren & Dobberstein, 1978) were added prior to incubation for 1 h at 37 °C. Reaction mixtures were then cooled quickly on ice, and 3 volumes of a buffer containing 10 mM NaH_2PO_4, pH 7.6, 1 mM EDTA, 1% (w/v) Nonidet P-40, 1% (w/v) sodium deoxycholate, and 0.3% sodium dodecyl sulfate ($NaDodSO_4$) was added, together with 2 μL of Antagosan (Behring), dithiothreitol to 2 mM, and unlabeled cysteine to 5 mM final concentrations. After 30 min at 0 °C, the reaction mixtures were centrifuged to remove undissolved membrane fragments, and to the supernatants was added 10 or 20 μg of cold competing peptide as indicated, together with approximately 50 μg of rabbit anti-SRIF IgG or an equivalent quantity of rabbit preimmune IgG.

After overnight incubation at 4 °C, followed by brief centrifugation in an Eppendorf centrifuge, supernatant fractions containing the immune complexes were isolated by using formaldehyde-fixed *Staphylococcus aureus* (Richter et al., 1980) or protein A–Sepharose CL-4B (Pharmacia) (see below). The immunoreactive products were dissolved in the electrophoresis sample buffer of Laemmli (1970) and analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis using a gradient running gel of 10–20% acrylamide. After fluorography (Bonner & Laskey, 1974), the dried gels were exposed to film (Kodak X-omat R) for 7–20 days.

Immunoreactive products first were isolated by using protein A–Sepharose to test whether or not the pro form synthesized in the presence of dog pancreas microsomal membranes was core glycosylated. Approximately 20 mg/50 μL of translation mixture of the dried affinity gel was reconstituted in successively 2 × 1 mL H_2O, then washed twice in 1 mL of 1 M acetic acid, twice in 1 mL of buffer A (20 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.5% Nonidet P-40, and 0.5 M NaCl), twice in the same buffer containing only 0.1 M NaCl (buffer B), and once in buffer C [10 mM NaH_2PO_4, pH 7.6, 1 mM EDTA, 1% (w/v) Nonidet P-40, and 1% (w/v) bovine serum albumin], and finally resuspended in 100 μL of the same buffer. This was then added to the supernatant fraction of the overnight immune reactions (see above), incubated with shaking at 4 °C for 30 min, and isolated by centrifugation. The protein A–Sepharose was then washed successively in 4 × 400 μL of buffer A, 2 × 400 μL of buffer B, and 400 μL of a buffer containing 150 mM NaCl and 10 mM sodium phosphate, pH 7.6. The bound immune complexes were eluted with a total of 600 μL of 1 M acetic acid. This eluate was frozen in liquid nitrogen and lyophilized overnight. The ability of the pro form thus isolated to bind to concanavalin A–Sepharose in the presence and absence of α-methyl mannoside (Sigma) was used as one test of glycosylation, with the methodology essentially being that of Colman et al. (1981). Alternatively, the altered migration of the pro form on $NaDodSO_4$–polyacrylamide gel electrophoresis after digestion by jack bean α-mannosidase was used. In this case the lyophilized pro form, isolated as above, was redissolved in 40 μL of 5 mM sodium acetate, pH 5.0, together with 1 μL of Antagosan and 10 μg of α-mannosidase (Boehringer) and incubated for 3 h at 37 °C. The unglycosylated preprosomatostatin was similarly treated to act as the control for possible proteolysis. After incubation, the reaction mixtures were frozen in liquid nitrogen, lyophilized, redissolved in electrophoresis sample buffer, and run on gradient gels as described above.

The in vitro biosynthesis of bovine arginine vasopressin/neurophysin II, as well as all other materials and methods not given in the text and figure legends, was as previously described (Richter et al., 1980).

Results

When bovine hypothalamic mRNA derived from membrane-bound polysomes is translated in a rabbit reticulocyte lysate system, using [^{35}S]cysteine as the radioactive label, only a single protein band, migrating with an apparent molecular weight (M_r) of 15 500, specifically reacts with antibodies against synthetic somatostatin (Figure 1, lane 4). The same precursor appears to be isolated by using two different antibody preparations (Figure 1, lanes 4 and 6). This protein band is not present on the autoradiograms, either when no exogenous mRNA is added to the system (Figure 1, lane 2), when preimmune IgG is used instead of anti-SRIF (Figure 1, lane 3), or when unlabeled somatostatin is added to the immune reaction as competing peptide (Figure 1, lane 5). The specificity of the immune reaction was further tested by competing the identified preprosomatostatin with a variety of other unlabeled peptides [20 μg each of neurophysins I and II, substance P, luteinizing hormone release hormone (all from Bioproducts), arginine vasopressin (Calbiochem), oxytocin (Ferring), insulin (Sigma), glucagon (Eli Lilly), β-melanotropin (Bachem, Switzerland), and β-endorphin (Serva)]; in no case was there a reduction in intensity of the somatostatin-specific band on the resulting autoradiograms (not illustrated). On translation

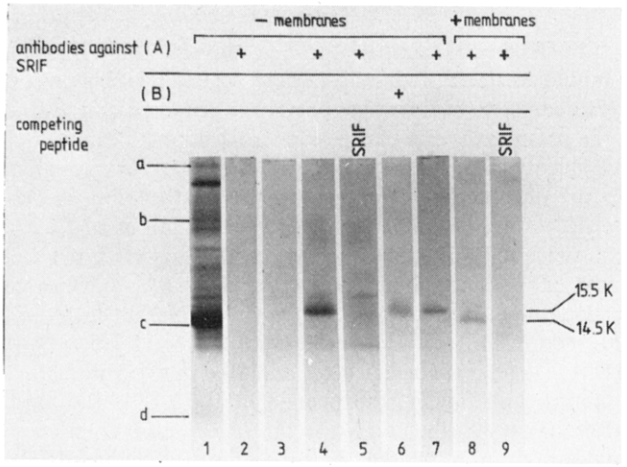

FIGURE 1: Translation of hypothalamic mRNA and immunoprecipitation with specific rabbit anti-SRIF IgG. (Lanes 1–6) Bovine mRNA (0.5 μg) per 25 μL of reaction mixture was translated in a rabbit reticulocyte lysate system, using 20 μCi of [^{35}S]cysteine (lanes 2–6) or 50 μCi of [^{35}S]methionine (lane 1) as radiolabel. The translation products were immunoprecipitated with 50 μg of anti-SRIF either from Bioproducts [(A) lanes 2, 4, and 5] or from Ferring [(B) lane 6]. Lane 1 shows 3 μL of a total [^{35}S]methionine-labeled translation before immunoprecipitation. In lane 2 no exogenous mRNA has been added; in lane 7, hypothalamic mRNA from rat was used. In lane 3, anti-SRIF was substituted by rabbit preimmune IgG. Lanes 8 and 9 are the same as lanes 4 and 5 but with cotranslational addition of 0.1 OD_{280} unit of dog pancreas microsomal membranes per 50 μL of reaction mixture. In lanes 5 and 9, 20 μg of unlabeled somatostatin was added during precipitation as competing peptide. Calibration proteins: (a) ovalbumin, M_r 46 000; (b) carbonic anhydrase, M_r 30 000; (c) lysozyme, M_r 14 300; (d) [^{125}I]Tyr-SRIF. Other details as under Materials and Methods.

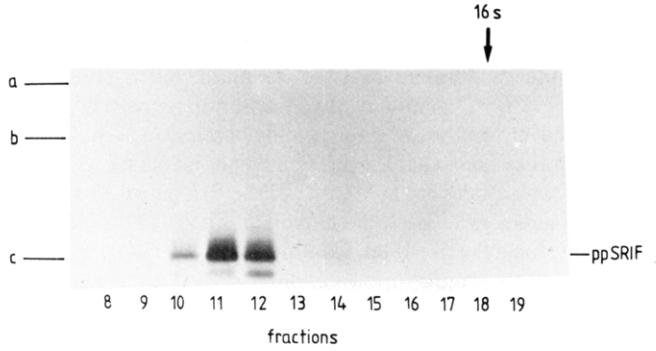

FIGURE 2: Immunoprecipitation of [^{35}S]methionine-labeled translation products of mRNA fractionated by sucrose density centrifugation. Poly(A+) bovine hypothalamic mRNA (30 μg) was layered onto a $NaDodSO_4$–sucrose gradient [15–35.9% (w/w)] and centrifuged at 40 000 rpm for 14 h at 21 °C in a Beckman SW40Ti rotor (Richter et al., 1980). Fractions of 0.35 mL were precipitated twice with ethanol at −20 °C and translated in 25 μL of rabbit reticulocyte translation mixtures with 50 μCi of [^{35}S]methionine as label. Immunoprecipitation was carried out by using Ferring anti-SRIF as given under Materials and Methods, and specific products were analyzed by $NaDodSO_4$–polyacrylamide gradient gel electrophoresis and fluorography (the smaller M_r ~12 000 protein in fractions 11 and 12 is not somatostatin specific). Calf thymus tRNA (4 S; fraction 5) and *E. coli* 16S (fraction 18) and 28S (fraction 24) ribosomal RNA were used as centrifugation standards. Electrophoresis calibration proteins as in Figure 1.

of mRNA from rat hypothalami, a similar M_r 15 500 precursor can be identified by the specific anti-SRIF antisera (Figure 1, lane 7).

The M_r 15 500 preprosomatostatin can be labeled not only with [^{35}S]cysteine but also with [^{35}S]methionine, but not with [^{125}I]Tyr-tRNA (New England Nuclear) included at 20 μCi/50 μL of translation mixture. On double labeling with [^{35}S]cysteine and [^{35}S]methionine and eluting the 15 500-dalton band from the electrophoresis gel with 96% efficiency, we have calculated that this somatostatin precursor accounts for not more than 0.15% of the total hypothalamic protein synthesized in vitro.

Since the M_r 15 500 preprosomatostatin must contain much sequence not corresponding to the tetradecapeptide hormone, it is natural in light of the precedent set by the opiocortin and neurophysin precursors to ask whether other functional entities may not also be included in the preprosomatostatin molecule. Recently it has been shown that the common prepro form to neurophysin I and to oxytocin, also synthesized in the hypothalamus, migrates at about M_r 16 500 (Schmale & Richter, 1980). To test whether this precursor might be identical with preprosomatostatin, double precipitation experiments were carried out, first reacting the translation products with anti-SRIF and subsequently with anti-neurophysin I (Bioproducts), and vice versa. The results (not shown) decisively indicate that these two precursors are discrete molecular species, with the first immune precipitation step having no influence on the subsequent one.

Poly(A+) RNA derived from bovine hypothalamus was further fractionated by sucrose density centrifugation (Richter et al., 1980). The size-graded mRNA fractions were then translated in a rabbit reticulocyte lysate system and the products reacted with anti-SRIF (Figure 2). The mRNA coding for the somatostatin precursor appears to be confined to a narrow size range (fractions 10–12) corresponding to a sedimentation value of 7–8 S.

Cotranslational addition of dog pancreas microsomal membranes gives rise to a pro form which migrates somewhat faster on $NaDodSO_4$–polyacrylamide gels, having an M_r of 14 500 (Figure 1, lane 8). Such microsomal membranes, in addition to containing a signal peptidase, are also able to core glycosylate a contemporarily translated precursor (Blobel & Dobberstein, 1975). Thus it is important to determine whether the M_r 14 500 prosomatostatin is also glycosylated. In Figure 3 are illustrated the results of binding experiments to concanavalin A–Sepharose. The M_r 14 500 prosomatostatin did not bind to the lectin, with there being no difference in elution pattern whether or not the concanavalin A–Sepharose was preabsorbed with α-methyl mannoside (Figure 3, lanes 7 and 8); pro-arginine vasopressin neurophysin II, known to be glycosylated (Schmale & Richter, 1981), is shown as the positive control (Figure 3, lanes 2–5). Unless the concanavalin A–Sepharose is preabsorbed with α-methyl mannoside (Figure 3, lane 3), this precursor binds to the lectin and is only eluted on washing with the sugar (Figure 3, lane 4). This result is confirmed in Figure 4, where treatment with jack bean α-mannosidase effects no change in apparent molecular weight, unlike in the positive control, the glycosylated pro-arginine vasopressin/neurophysin II (Figure 4, lane 5). These results strongly suggest that the M_r 14 500 prosomatostatin is not glycosylated and thus that the small change in molecular weight on cotranslational addition of microsomal membranes is probably attributable to the loss of a short signal sequence of about 10 amino acids.

Discussion

The results presented here show that mRNA derived from membrane-bound polysomes of bovine hypothalami can be translated in a rabbit reticulocyte lysate system to yield among the translation products a somatostatin precursor of M_r 15 500. The identification of this putative preprosomatostatin was effected by two different antisera raised against the synthetic tetradecapeptide. Furthermore, only the synthetic somatostatin

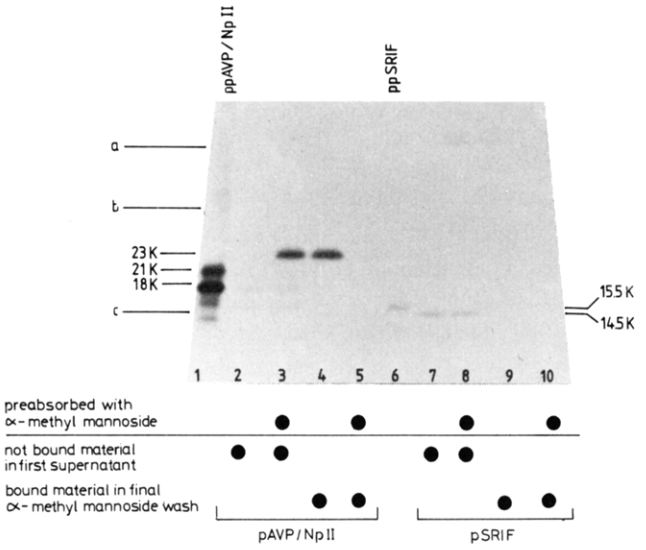

FIGURE 3: Concanavalin A affinity chromatography of prosomatostatin. Prosomatostatin (pSRIF; lanes 7–10) and pro-arginine vasopressin/neurophysin II (pAVP/NpII; lanes 2–5) were synthesized in 50 μL of [^{35}S]cysteine-labeled rabbit reticulocyte translation mixtures supplemented with dog pancreas microsomal membranes, immunoprecipitated, and isolated by using protein A–Sepharose. The precursors were incubated with gentle rotation at room temperature each with 100 μL of prewashed concanavalin A–Sepharose suspension in a binding buffer containing 150 mM NaCl, 0.7 mM $MgCl_2$, 1 mM dithiothreitol, 0.7 mM $MnCl_2$, 0.7 mM $CaCl_2$, 0.05% (w/v) sodium dodecyl sulfate, and 20 mM Tris-HCl, pH 7.5, in the absence (lanes 2, 4, 7, and 9) or presence (lanes 3, 5, 8, and 10) of 0.4 M α-methyl mannoside. After 30 min, the tubes were centrifuged and these first supernatants, containing unbound proteins, retained (lanes 2, 3, 7, and 8). The concanavalin A–Sepharose beads were then washed in 3 × 1 mL of respective binding buffer; for elution of any bound glycoproteins, the affinity beads were finally washed in binding buffer plus 0.4 M α-methyl mannoside (final α-methyl mannoside wash; lanes 4, 5, 9, and 10). All eluates were precipitated by addition of cold trichloroacetic acid to 10% (w/v) in the presence of 20 μg of bovine serum albumin carrier, and after centrifugation, the pellets were washed once in 0.5 mL of cold (–20 °C) acetone, before being recentrifuged, dried, electrophoresed, and fluorographed. The respective prepro forms, shown in lanes 1 and 6, were obtained by immunoprecipitation of translations carried out in the absence of microsomal membranes and were not subject to concanavalin A–Sepharose affinity chromatography. Calibration proteins are as in Figure 1; all other details are given under Materials and Methods.

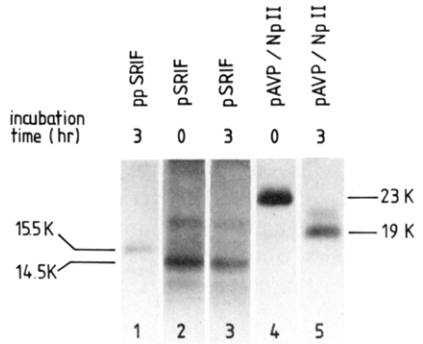

FIGURE 4: Treatment of prosomatostatin with jack bean α-mannosidase. [^{35}S]Cysteine-labeled prosomatostatin (lanes 2 and 3) or pro-arginine vasopressin/neurophysin II (lanes 4 and 5) was immunoprecipitated, isolated via protein A–Sepharose, and incubated with α-mannosidase, as described under Materials and Methods, at 37 °C for the times indicated. (Lane 1) Preprosomatostatin also treated with α-mannosidase.

and no other peptide tested could compete out this M_r 15 500 precursor. No cross-reactivity could be detected between these antisera and peptides such as neurophysins I and II and arginine vasopressin as has been reported by others (van Leeuwen et al., 1979). The same antisera also are able to recognize a protein translated from rat hypothalamic mRNA (Figure 1, lane 7), running at the same apparent molecular weight on $NaDodSO_4$–polyacrylamide gel electrophoresis, which is probably identical with the M_r 15 000 preprosomatostatin identified by hybridization of the specific mRNA with a synthetic gene by Joseph-Bravo et al. (1980). The size of the mRNA identified by this probe corresponds well with the estimated sedimentation coefficient determined here from sucrose density centrifugation (Figure 2).

The presence of dog pancreas microsomal membranes during the in vitro translation yields an unglycosylated precursor of M_r 14 500. This prosomatostatin is compatible with the several large molecular weight species isolated from a variety of mammalian tissues, the majority of which have values between 12 000 and 15 000 daltons (Spiess & Vale, 1978, 1980; Lauber et al., 1979; Patzelt et al., 1980). It also suggests a rather short signal sequence of about 10 amino acids. Precise confirmation of the length of the signal sequence will only be obtained by comparing the amino acid sequences of the prepro and pro forms of the hormone, an approach which is precluded at the present time by the very small quantities of these precursors that can be synthesized. Two different tests of glycosylation were used here, both of which are reliable probes of the mannose-rich core invariably associated with glycosylation of secretory proteins by endoplasmic reticulum (Waechter & Lennarz, 1976). Both tests were negative. Unfortunately, no information is available either on the length of the signal sequence or on glycosylation in other mammalian somatostatin precursors. Patzelt et al. (1980) detected mannose and glucosamine incorporation into a protein which comigrated with prosomatostatin synthesized in vivo in rat pancreatic islets, but tunicamycin treatment had no influence on these sugars, and this result is thus inconclusive.

A comparison between the mammalian and fish somatostatin precursors suggests that the two classes are not directly comparable: the fish precursors have a longer signal sequence (ca. 25 amino acids; Goodman et al., 1980a,b; Hobart et al., 1980; Shields, 1980) and in the N-terminal cryptic region show little homology with the sequences of the N-terminally extended 25 and 28 amino acid somatostatins from mammalian tissues (Pradyrol et al., 1978; Böhlen et al., 1980; Schally et al., 1980; Spiess et al., 1981). It also remains to be seen whether or not, like fish, mammals have multiple genes for somatostatin.

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Effect of pH and Fatty Acid Chain Length on the Interaction of Myelin Basic Protein with Phosphatidylglycerol†

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ABSTRACT: The basic protein of myelin binds electrostatically to acidic lipids but has several hydrophobic segments which may penetrate into the lipid bilayer. Calorimetric and spin-label evidence suggests that below the phase transition temperature, T_c, several phase states occur in the complex of phosphatidylglycerol with basic protein, possibly due to differences in the degree of penetration of the protein and/or interdigitation of the lipid acyl chains. One of these states is a metastable state which starts to melt 10 °C below the T_c of the pure lipid and then refreezes, with release of heat, into a stable state. The stable state melts near the T_c of the pure lipid but restricts the motion of a fatty acid spin-labeled near the terminal methyl much more than does the pure lipid. The relationship between the rate of conversion to the stable state and the degree of penetration of the protein at varying pH, in the range 4-8, and the lipid acyl chain length, in the range 14 to 18 carbons, was investigated. Altering the pH in this range affects protonation of the histidines of the protein but has no effect on the lipid at pH 4 and above. The rate of conversion of the sample to both the metastable state and the stable state decreased with increase in pH for phosphatidylglycerol with all lipid chain lengths. It also decreased with decreasing chain length at constant pH. This suggested that the lipid could refreeze into the stable state more readily if a smaller proportion of the total bilayer thickness was occupied by the hydrophobic segments of the protein. The consistency of these results with the concept of penetration of portions of the protein partway into the bilayer lends support to this hypothesis.

The basic protein of central nervous system myelin contains 10 histidines, 13 lysines, and 18 arginines and interacts electrostatically with acidic lipids (Palmer & Dawson, 1969; Demel et al., 1973; London et al., 1973; Boggs & Moscarello, 1978). However, it also contains several segments composed of 5-10 hydrophobic and neutral amino acids which may penetrate partway into the hydrocarbon region of the bilayer. This conclusion is based on the perturbing effects of the protein on the lipid (Gould & London, 1972; Papahadjopoulos et al., 1973, 1975; Demel et al., 1973; Boggs & Moscarello, 1978; Boggs et al., 1980, 1981a) as well as evidence for sequestration

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