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ISOLATION AND PARTIAL CHARACTERIZATION OF A BIOSYNTHETIC N-TERMINAL METHIONYL PEPTIDE OF BOVINE PARS INTERMEDIA: RELATIONSHIP TO UBIQUITIN

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SUMMARY. During in vitro labelling studies of beef pituitary intermediate lobe cells, a 4000 daltons molecular weight peptide was found to be biosynthesized in major yields. The partial amino acid sequence of this peptide has been found to be Met 1, Leu 8, 15 and Lys 6, 11, 27, 29, 33. This partial sequence fits very well the one expected from the N-terminal sequence of bovine and human ubiquitin, a non-histone fragment of the nuclear protein A24. Since an identical peptide was also biosynthesized from rat hypothalamus and mouse AtT-20 tumor cells, the ubiquitous nature of this peptide is further revealed.

INTRODUCTION

The pars intermedia of pituitary glands secretes relatively large amounts of LPH*, ACTH** and related fragments (1,2). It was shown recently by immunohistochemical studies that beta-LPH and ACTH are uniformly distributed in all secretory cells of the pars intermedia in a number of species (3). Hence this tissue represents a good model in which the biosynthesis of beta-LPH, ACTH and their related fragments can be investigated (4-8).

* LPH: Lipotropic hormone, beta-lipotropin
** ACTH: Adrenocorticotropic hormone.

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During the course of our investigations on such biosynthesis in bovine pars intermedia, we obtained a pure radiolabelled peptide of about 4,000 daltons molecular weight, which seems to be one of the major biosynthetic products after 3 hrs incubations of neurointermediate lobe cells (7,8). The partial sequence of this peptide labelled with (^{35}S)Met, (^{3}H)Lys and (^{3}H)Leu revealed an amino acid sequence which was unrelated to either beta-LPH, ACTH or any of their segments. It was thus decided to look for a candidate which could fit its molecular sequence. By performing a computer data search to possibly match this partial sequence with that of any known protein or segment of protein, it was possible to find identical sequence positions of all eight methionine, lysine and leucine amino acids positioned within the first 33 residues of a recently characterized (9-11) thymic non-histone fragment of the nuclear protein A-24 complex, named ubiquitin (11,12). This paper will deal with the biosynthesis isolation and partial characterization of this peptide from bovine pars intermedia and the likelihood of it being ubiquitin or an N-terminal fragment of it.

MATERIALS AND METHODS.

The pars nervosa and pars intermedia were carefully dissected from the anterior lobe of 25 fresh beef pituitary glands. The cells of the pars intermedia were then dispersed by gentle agitation and were suspended in 10 ml of ice cold Krebs-Ringer (13) solution containing 0.2% glucose (KRBG)*. After an initial preincubation period of one hour, the cells were incubated for 3 hours in a KRBG solution containing either 5 mCi ^{3}H-leucine or a mixture of 1 mCi ^{35}S-methionine and 4.8 mCi ^{3}H-lysine. The cells were then homogenized and extracted in 5 ml of 10^{-3} M EDTA (pH 10.35) solution containing 5 mg/ml sheep pituitary fraction D (7). After desalting the extract was chromatographed together with 200 mg of fraction D on carboxymethyl cellulose column (1 x 40 cm).

Disc electrophoresis of carboxymethyl cellulose chromatography fractions were performed on polyacrylamide gels at pH 4.5 or pH 8.3 according to Reisfield et al. (14) and Davis et al.

*KRBG: Krebs-Ringer bicarbonate glucose.

(15). The gels were cut into 2 mm slices and digested overnight at 50°C in 30% H_2O_2 and the radioactivity was counted in 10 ml of a triton X-100 Toluene (1:2 V/V) scintillation cocktail containing 5.6 g Omnifluor/l (New England Nuclear).

Automatic Edman degradation of the purified labelled peptides was performed on a Beckman 890B sequencer, using 150 mmoles of sperm whale apomyoglobin as carrier and 0.1 M QUADROL buffer (16). The thiazolinones collected in butyl chloride were counted directly in a toluene base scintillation mixture (4 g Omnifluor/l Toluene).

RESULTS.

The carboxymethyl cellulose chromatography on the desalted supernatant of cells homogenate is shown in Fig. 1. The fractions 59-77, 195-235 and 236-270 were shown previously (7) to contain gamma-LPH, beta-LPH and beta-endorphin respectively. The vast majority of the radioactivity bound to the CMC column was found in fractions 110-140 (Fig. 1) and the material obtained after lyophylization of this fraction was further characterized as follows:

1. The purity of the radiolabelled material was confirmed by polyacrylamide gel electrophoresis at both pH 4.5 and 8.3, as shown in Fig. 2. It is seen that the radiolabelled peptide migrates as a single band at both pH's with an Rf of 0.59 and 0.22 respectively.

2. On a column of Sephadex G-25 (1.7 x 60 cm) the peptide elutes in the void volume. The molecular weight was then determined on a calibrated Sephadex G-75 column (1.7 x 60 cm) whereby it coelutes with standard sheep beta-endorphin (20) and an apparent molecular weight of about 4,000 daltons was calculated.

Upon SDS-polyacrylamide electrophoresis (17), this radiolabelled peptide migrated close to the marker (Fig. 3a), hence showing a molecular weight below 10,000 daltons. Using the SDS/urea-polyacrylamide electrophoresis (18) designed for molecular weight estimation in the range of 10,000 to 2,000, this peptide comigrated with $beta_{sheep}$-endorphin, of molecular weight 3,500

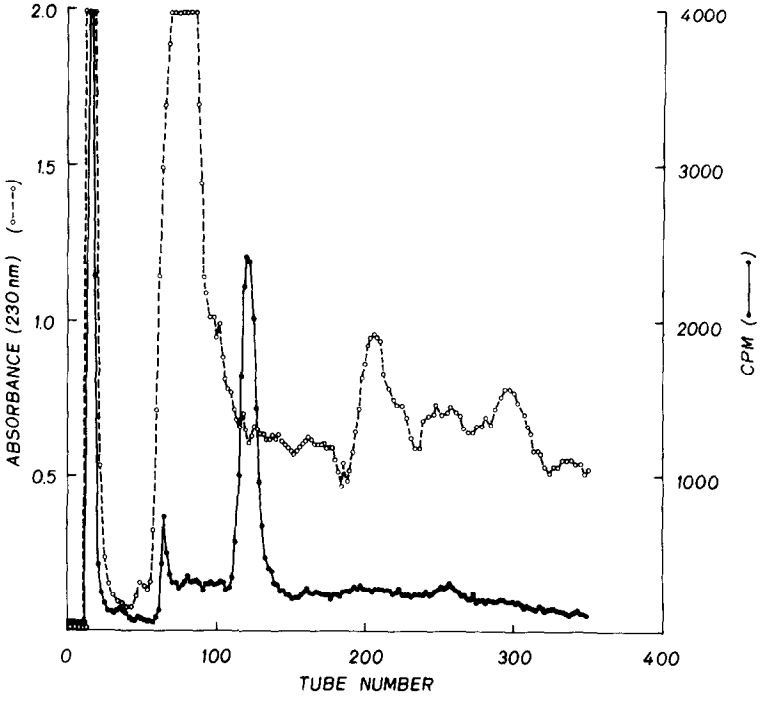

Fig. 1. CM-cellulose chromatography of labelled (^{35}S-Met, ^{3}H-Lys) proteins extracted from the isolated cells of beef pars intermedia, incubated in vitro for 3 hours. The elution was performed using NH_4OAc gradients as described previously (6-8). A similar pattern was obtained with ^{3}H-Leu labelled proteins.

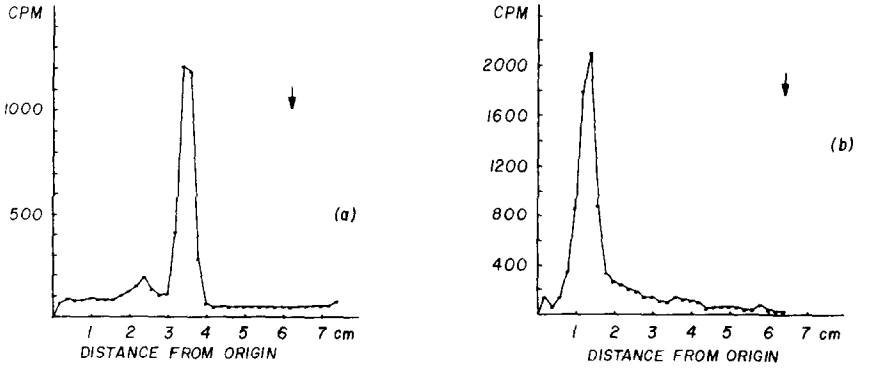

Fig. 2. Polyacrylamide gel electrophoresis at pH 4.5 (a) and pH 8.3 (b) on the radioactive material recovered in fraction (110-140) of the CM-cellulose chromatography (Fig. 1). The arrow shows the position of the tracking dye at the end of the migration.

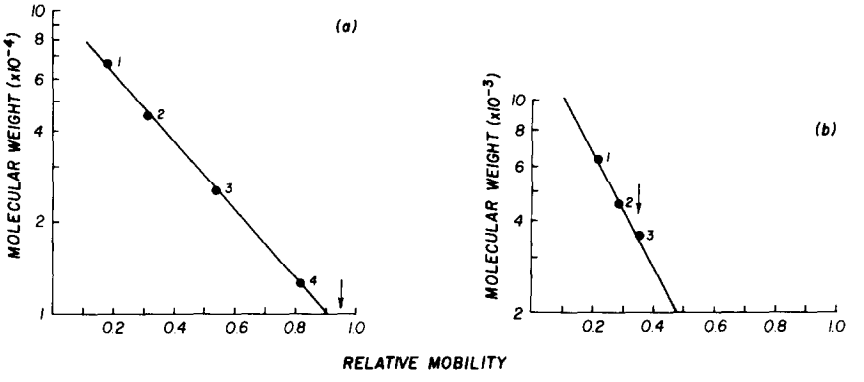

Fig. 3a. Sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis (16) of the material in fraction (110-140). The mobility was calculated relative to that of bromophenol blue (mobility - 1.0). The arrow indicates the relative mobility of this peptide. The standard curve was calculated from the positions of (1) bovine serum albumin (2) hen egg albumin (3) chymotrypsinogen and (4) cytochrome C of molecular weights 67,000; 45,000; 25,000 and 12,500 respectively.

3b. SDS/urea polyacrylamide gel electrophoresis of fraction (110-140) at pH 6.8 using 12.5% acrylamide gels with a ratio of (1:10) acrylamide:N,N'methylenebis acrylamide (17). The arrow indicates the relative mobility of this peptide. The standard curve was drawn from the positions of (1) gamma-lipotropin (6,350), (2) sheep ACTH (4,540), (3) sheep beta-endorphin (3,325), peptides routinely purified in this laboratory.

(19,20) (Fig. 3b). Since the reliability of the molecular weights deduced by this method is close to $\pm$ 20% (18), the value obtained is thus in good agreement with that deduced from the molecular sieving method on Sephadex G-75.

3. The results of the sequence of (^{35}S) Methionine and (^{3}H) Lysine labelled peptide are shown in the upper two graphs of Fig. 4. It is seen that methionine occupies the amino terminal residue and is not found anywhere else up to residue 40. Lysine occupies positions 6, 11, 27, 29 and 33 within the first 40 residues of the polypeptide chain.

4. The (^{3}H) Leucine labelled peptide similarly obtained was sequenced and showed that leucine occupies positions 8 and 15 within the first 40 amino acids.

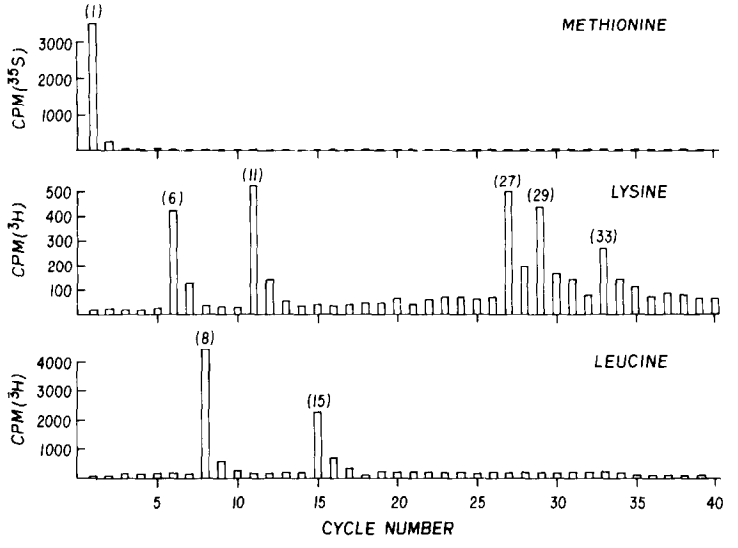

Fig. 4. Partial amino terminal sequence analysis of radioactive peptide in fraction (110-140). Total input of radioactivity was 5 x 10^3 ^{35}S-Met CPM, 4.5 x 10^3 ^{3}H-Lys CPM and 1 x 10^4 ^{3}H-Leu CPM.

With such a partial sequence at hand, we asked Dr Margaret Dayhoff to perform a computer data search on the likelihood that this sequence would be present within the sequence of any known molecule. Unexpectedly, an excellent fit (21) of all placed eight residues within the amino terminal sequence of ubiquitin (10-12) originally extracted and characterized from bovine and human thymus and called ubiquitous immunopoietic polypeptide, UBIP (9-11). Moreover in ubiquitin, no methionine, leucine or lysine appear at any other positions between residues 1 to 40, in agreement with our sequence results (Fig. 4).

DISCUSSION.

The results presented in this paper, clearly shows that the cells of the neurointermediate lobe of bovine pituitaries, actively biosynthesize a peptide with a partial sequence Met 1, Leu 8, 15 and Lys 6, 11, 27, 29, 33. When performing a protein data

search on the sequence similarity of this peptide with that of other bovine proteins, the results of the data collection showed (Dayhoff, M.O., personal communication) (21):

1. The total number of sequences searched was 1050 and the total number of segments of length 33 amino acids compared to the test peptide was 84,889. In this whole computer search for segments with methionine, leucine and lysine at the positions found (Fig. 4), ubiquitin alone matches all eight positions. Furthermore ubiquitin does not contain any Methionine, Leucine or Lysine at any other positions between residues 1-40. No other segment of any protein scored more than 5 matches and all with a score of 5 contain other Methionine, Leucine or Lysine at other positions within their 33 amino acid segment. Clearly ubiquitin matches much better than any chance identity expected from the distribution of other matches.

2. A more complicated scoring system was also used (12, 21) which better utilizes the information and from which a probability that two sequences could be identical over a region of 33 consecutive residues could be calculated. In such a system a histogram is plotted for the matching score of all segments analyzed as a functio of the number of matching segments with that score. A normal distribution of matching scores is thus obtained. This showed a minimum score of 45 and a maximum score of 74 (21). The average score was 54.937 and the standard deviation (SD) was 2.4524, with 84,889 comparisons. Our test peptide, gave a score of 74, i.e., 7.7 SD above the mean for the normal distribution. The probability of obtaining a similar score from any other segment is $P = 3 \times 10^{-15}$. Thus our sequence must be part of ubiquitin or a closely related gene product. To our knowledge this type of computer data search on a partially

sequenced protein with distantly placed residues has never been done before (Dayhoff, M.O., personal communication).

The next question to arise is the disparity between the molecular weight we find (4,000 daltons) and the 8,500 daltons reported for the 74 amino acid ubiquitin (9-12). Since these molecular weights differ by a factor of two, it seems likely that our biosynthetic peptide represents a fragment of ubiquitin. This puzzling result will have to be resolved by comparing the properties of the native peptide with our biosynthetic product, both on sephadex and polyacrylamide urea/SDS gels.

Recent results in this laboratory have shown that a biosynthetic peptide of identical sequence and properties on polyacrylamide gels is also obtained when labelling cells of rat hypothalamus and of mouse AtT-20 tumor (25). The presence of this peptide in three different tissues and two mamalian species and the constancy of its sequence is in agreement with the results obtained in other laboratories (9-12, 22), where it was shown to be immunologically similar in many tissues such as thymus, liver, kidney, salivary gland, etc... and in as far related species as bovine and celery and may be a universal content of living cells (22).

Although ubiquitin induces the differentiation of T (thymus derived) lymphocytes and B (bone-marrow derived) lymphocytes in vitro (22,24) activates adenylate cyclase in a variety of tissues (24) probably via beta-adrenergic receptors and seems to be part of the nonhistone component of the nuclear protein A24 complex (12), its basic physiological function is still unknown The kinetics of its maturation will have to await pulse-chase labeling techniques, currently in progress in this laboratory.

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REFERENCES

1. LaBella, F., Queen, G., Senyshyn, J., Lis, M. and Chrétien, M. (1977), Biochem. Biophys. Res. Commun. 75, 350-357.
2. Moriarty, G.C. (1973), J. Histochem. Cytochem. 21, 855-894.
3. Pelletier, G., Leclerc, R., Labrie, F., Côté, J., Chrétien, M. and Lis, M. (1977), Endocrinology 100, 770-776.
4. Bertagna, X., Lis, M., Gilardeau, C. and Chrétien, M. (1974), Can. J. Biochem. 52:349-358.
5. Chrétien, M., Lis, M., Gilardeau, C. and Benjannet, S. (1976) Can. J. Biochem. 54, 566-570.
6. Crine, P., Benjannet, S., Seidah, N.G., Lis, M. and Chrétien, M. (1977), Proc. Natl. Acad. Sci. USA 74, 1403-1406.
7. Crine, P., Benjannet, S., Seidah, N.G., Lis, M. and Chrétien, M. (1977), Proc. Natl. Acad. Sci. USA 74, 4276-4280.
8. Crine, P., Benjannet, S., Seidah, N.G., Lis, M. and Chrétien, M. (1977). Fifth American Peptide Symposium, LaJolla, California, June 20-24, In Press.
9. Goldstein, G., Scheid, M., Hammerling, U., Boyse, E.A., Schlesinger, D.H. and Niall, H.D. (1975), Proc. Natl. Acad. Sci. USA 72, 11-15.
10. Schlesinger, D.H., Goldstein, G. and Niall, H.D. (1975) Biochemistry 14, 2214-2218.
11. Schlesinger, D.H. and Goldstein, G. (1975), Nature 255, 423-424.
12. Hunt, L.T. and Dayhoff, M.O. (1977), Biochem. Biophys. Res. Commun. 74, 650-655.
13. Krebs, H. (1950), Biochim. Biophys. Acta 4, 249-253.
14. Reisfield, R.A., Lewis, V.J. and Williams, D.E. (1962) Nature 195, 281-283.
15. Davis, B.J. (1965), Ann. N.Y. Acad. Sci. 121, 404-427.
16. Brauer, A.W., Margolies, M.N. and Haber, E. (1975) Biochemistry 14, 3029-3031.
17. Weber, K. and Osborn, M. (1969), J. Biol. Chem. 244, 4406-4412.
18. Swank, R.T. and Munkres, K.D. (1971) Anal. Biochem. 39, 462-477.
19. Chrétien, M. and Li, C.H. (1967), Can. J. Biochem. 45:1163-1174.
20. Chrétien, M., Benjannet, S., Dragon, N., Seidah, N.G. and Lis, M. (1976), Biochem. Biophys. Res. Commun. 72:472-478.
21. Dayhoff, M.O., Hunt, L.T., Baker, W.C. and Schwartz, R.M. (1977), Protein Sequence Data File, National Biomedical Research Foundation, Washington D.C.
22. Goldstein, G. (1974), Nature 247, 11-14.
23. Baseh, R.S. and Boldstein, G. (1974), Proc. Natl. Acad. Sci. USA 71, 1474-1478.
24. Goldstein, G., Scheid, M., Hammerling, U., Boyse, E.A., Schlesinger, D.H. and Niall, H.D. (1975), Proc. Natl. Acad. Sci. USA 72, 11-15.
25. Seidah, N.G., Benjannet, S., Scherrer, H., Crine, P. and Chrétien, M.: (Manuscript in Preparation).