NEUROPHYSIN METHYLATION IN EXTRACTS OF BOVINE POSTERIOR PITUITARY GLAND: HORMONE BINDING ABILITY

David H. EDGAR and Derek B. HOPE
University Department of Pharmacology, South Parks Road, Oxford, OX1 3QT, UK

Received 14 October 1974
Revised version received 28 October 1974

1. Introduction

The highest specific activity of protein carboxymethylase (*S*-Adenosyl-L-methionine : protein *O*-methyltransferase, EC 2.1.1.24) is found in the pituirary gland [1]. Both lobes of the gland contain the enzyme although the posterior lobe appears to have the greater activity [2]. Recently, it was reported that six of the anterior pituitary hormones were good substrates for pituitary carboxymethylase [1]. The posterior lobe hormones, oxytocin and vasopressin, were included in this study but since they lack a free carboxyl group they were not methylated.

Together with the high protein carboxymethylase activity, extracts of posterior pituitary glands contain endogenous methyl-acceptor(s) [3]. In the present communication evidence is presented which is consistent with the identification of neurophysin as a major acceptor. The ability of agarose-coupled [8-lysine]-vasopressin to bind methylated and unmodified protein with similar avidity is discussed.

2. Materials and methods

2.1. Preparation of extract

Acetone-dried bovine posterior pituitary gland powder (Paines and Byrne Ltd., Greenford, UK) was extracted at 4°C with a pH 6.0 buffer, containing 50 mM sodium phosphate, 5 mM disodium EDTA and 2.5 mM 2-mercaptoethanol. After 18 hr the mixture was centrifuged at 15 000 *g* for 40 min and the supernatant dialysed against the same buffer for 6 hr before recentrifugation. Protein in the supernatant extract was determined [4] and the extract stored at −20°C.

2.2. Methylation

The methylation reaction mixture contained 10 μM *S* adenosyl-L-[^{14}C]methyl methionine (specific activity 55 mCi/mmol; Radiochemical Centre, Amersham, UK) plus pituitary extract containing 200 μg protein, in the pH 6.0 buffer to a final volume of 200 μl. Following incubation at 37°C for 40 min, the reaction was terminated by addition of 200 μl ice-cold 0.1 M formic acid.

Protein-bound radioactivity was separated from low molecular weight radioactive materials by gel-filtration through a column of Sephadex G25 (Pharmacia G.B. Ltd., UK), using 50 mM formic acid as eluent at 4°C. Fractions containing radio-activity excluded from the gel were pooled, lyophilized and stored at −20°C.

2.3. Affinity chromatography

A crude mixture of bovine neurophysins obtained from Sephadex G75 gel filtration [5] was added in excess to the labelled protein excluded from Sephadex G25, in order to minimise losses of radioactivity during subsequent steps. This was then eluted in pH 5.8 potassium phosphate buffer through a column of [8-lysine]-vasopressin bound to agarose. Coupling of vasopressin via the ε-amino group of lysine to cyanogen bromide-activated Sepharose 4B (Pharmacia G.B. Ltd., UK) was achieved by reversibly protecting the α-amino group of the vasopressin [6] by formation

of a vasopressin–acetone complex [7]. Protein binding at pH 5.8 to the agarose-coupled vasopressin was eluted with 50 mM formic acid.

2.4. SDS gel electrophoresis

Polyacrylamide gel electrophoresis was performed using 12.5% (w/v) 'Cyanogum 41' (B.D.H. Ltd., UK) in a pH 6.5 buffer containing 0.1% (w/v) sodium dodecyl sulphate [8]. Protein bands were fixed and stained with 0.1% (w/v) amido black in 25% (w/v) trichloroacetic acid.

2.5. Measurement of radioactivity

A Beckmann LS200B liquid scintillation counter was used to estimate radioactivity. Aqueous samples were counted in 67% (v/v)toluene–33%(v/v) Triton X100 containing 0.5% (w/v) PPO and 0.01% (w/v) POPOP.

3. Results and discussion

After completion of the incubation, 15% of the methyl label was incorporated into molecules of mol. wt. greater than 5 000, being excluded from Sephadex G25. On SDS-poly-acrylamide gel electrophoresis, the methylated protein co-migrated with bovine neurophysin. Following the method of Paik et al. [9], treatment with 0.5 M borate buffer, pH 8.4, for 2 hr at room temperature resulted in loss of 95% protein-bound label, the activity becoming volatile and so removable by lyophilization.

These results, considered in conjunction with the ability of 75% of the methylated protein to bind specifically at pH 5.8 to agarose-coupled vasopressin (fig. 1), indicate that carboxylic acid moieties of the bovine neurophysin present in the extract had been carboxymethylated. The nature of the radioactive

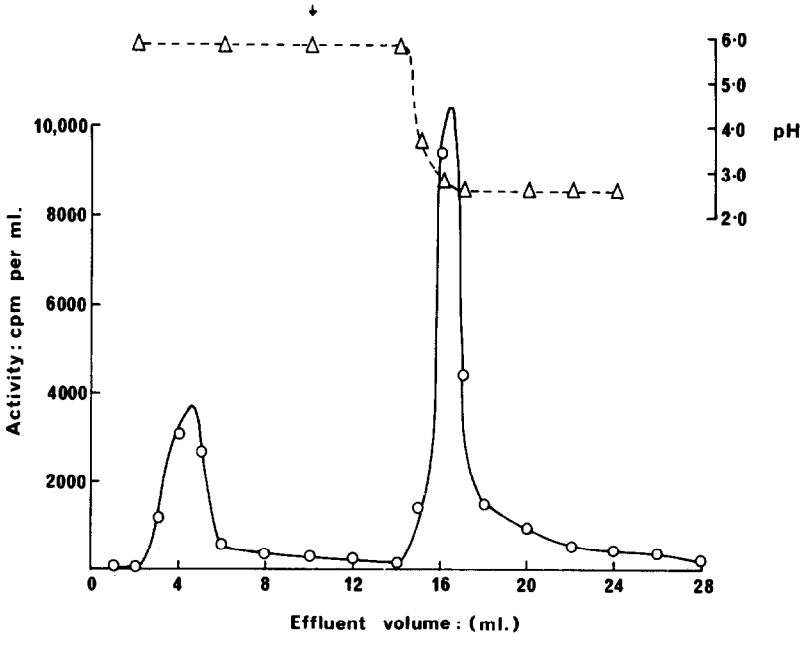

Fig. 1. Elution profile of protein-bound label after affinity chromatography on agarose-coupled vasopressin. 150 μg labelled protein, excluded from a Sephadex G25 column, was added to 7 mg bovine neurophysin in 1.5 ml 0.1 M phosphate buffer, pH 5.8. The solution was applied to the top of a 1 cm × 5 cm column of agarose-coupled vasopressin, equilibrated in the same buffer. The flow-rate was 12 ml/hr, and 1 ml samples were collected. Arrow shows where 50 mM formic acid was substituted for the buffer.

species not bound to the column is being investigated. The labelled protein, which bound to the agarose-coupled vasopressin at pH 5.8, could be eluted from the gel along with added unmethylated bovine neurophysin by either acid (pH 2.5) or alkaline (pH 8.5) conditions. However, as the methyl ester is unstable even at mildly alkaline pH, the elution of methylated neurophysin was routinely achieved with 50 mM formic acid. Further confirmation that neurophysin is an effective acceptor for pituitary carboxymethylase was produced after completion of this work [10].

The relative abilities of methylated and unmodified neurophysins to bind-agarose-coupled vasopressin were then investigated. Some difference of binding characteristics was expected, as methylation, by neutralization of side-chain acidic residues should have a profound effect on the nature of the molecule with consequent alteration of hormone-binding ability. Fig. 2 shows, however, that the methylated and unmodified bovine neurophysins, obtained previously by acid-elution of agarose-coupled vasopressin, compete with equal avidity for limited amounts of hormone.

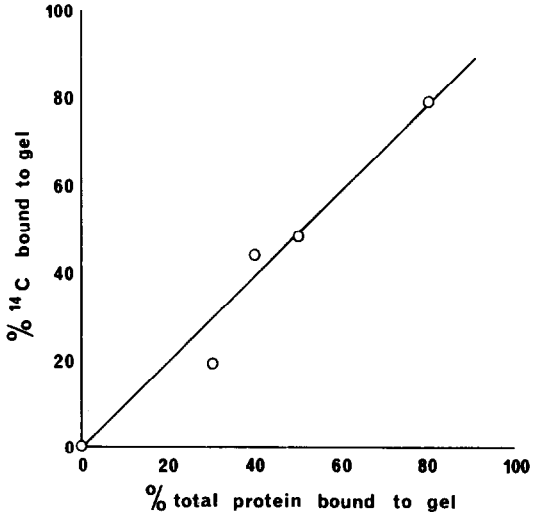

Fig. 2. Binding of label and total protein to agarose-coupled vasopressin. 100 μg labelled protein and 4.8 mg bovine neurophysin, together recovered from the affinity column (fig. 1) after acid elution, were dissolved in 10 ml 0.1 M potassium phosphate buffer, pH 5.8. The solution was titrated with increasing amounts of agarose-coupled vasopressin. Label and total protein in the supernatant were measured after each addition of gel. The amount of gel-bound label and protein was calculated.

Previous work [11] has indicated that hormone-neurophysin binding involves an electrostatic interaction between the α-amino group of the hormone and an unprotonated carboxylic acid moiety in the protein, although some secondary non-electrostatic interaction between hormone and neurophysin has been demonstrated. The fact that methylation does not appear to change the hormone-binding ability of neurophysin would argue against methylation of that carboxylic acid moiety required for normal binding. Thus the specificity of the enzyme is such that the carboxyl group necessary for hormone binding is not the primary acceptor.

The extent of any in vivo methylation has yet to be demonstrated. It is possible, however, that although the degree of enzymic methylation produced in these experiments has no effect on hormone-binding, a more extensive methylation of neurophysin might change the binding characteristics.

It is interesting tot note that chemical modification of carboxylic acid groups of ovine adreno-corticotrophic hormone with glycine methyl ester or taurine, while not changing lipolytic activity of the hormone completely destroyed the adrenocorticotrophic activity [12]. Perhaps methylation of neurophysin, here shown not to change hormone-binding ability, effects a change in some other biological property of the protein.

Acknowledgements

This work was supported by a Medical Research Council grant. D.H.E. is a Christopher Welch Scholar.

References

[1] Diliberto, E. J. and Axelrod, J. (1974) Proc. Natl. Acad. Sci. U.S., 71, 1701.
[2] Axelrod, J. and Daly, J. (1965) Science, 150, 892.
[3] Morin, A. M. and Liss, M. (1973) Biochem. Biophys. Res. Commun. 52, 373.
[4] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265.
[5] Hollenberg, M. D. and Hope, D. B. (1967) Biochem. J. 104, 122.
[6] Robinson, I. C. A. F. and Walker, J. M. (1974) J. Endrocrinol. In press.

[7] Yamashiro, D., Havran, R. T., Aanning, H. L., du Vigneaud, V. (1967) Proc. Natl. Acad. Sci. U.S., 57, 1058.
[8] Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406.
[9] Paik, W. K., Lee, H. W. and Morris, H. P. (1972) Cancer Res. 32. 37.
[10] Axelrod, J. (1974) The International Conference on Neurophysin Proteins. New York Acad. Sci. To be published.
[11] Breslow, E. (1974) Advances in Enzymology, 40, 271.
[12] Gilardea, C., Chrêtien, M. and Lis, M. (1974) Can. J. Physiol. Pharmacol. 52, 723.