COVALENT BINDING OF [8-LYSINE]-VASOPRESSIN TO HIGH-MOLECULAR-WEIGHT CARRIERS

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A commercial preparation of [8-lysine]-vasopressin was purified by chromatography and free-flow continuous electrophoresis; the procedure yielded 50—60% of product with pressor activity of 240—260 I.U./mg. The formation of a covalent bond between the hormone and protein carriers (bovine serum albumin and porcine immunoglobulin) or poly-L-glutamic acid was determined by using tritium-labelled [8-lysine]-vasopressin as a marker. The peptide bond was synthetized by means of carbodiimides or Woodward's reagent. The effect of salts, the ratio of the individual components and pH on the amount of hormone bound to the carrier was investigated.

High-molecular-weight conjugates of neurohypophysial hormones were used as antigens. Methods for the formation of bonds between a hormone and various carriers have been reported[1,2]. The present work aimed at determining the conditions under which a covalent bond was formed between LVP** and a high-molecular-weight carrier in the presence of two condensation reagents — carbodiimide and Woodward's reagent. Bovine serum albumin (BSA), porcine immunoglobulin (pIgG) and poly-L-glutamic acid (poly-Glu) were used as carriers. An investigation was made of the conditions (pH, ratio of reaction components, duration of reaction and temperature) necessary for the formation of a covalent bond between the hormone and carrier. [^{3}H]-LVP was used as a marker for determining the amount of bound hormone.

The preparation of high-molecular-weight conjugates with neurohypophysial hormones in sufficient amounts is possible only if a greater quantity of pure hormone is available. We present a method for the isolation of LVP from a crude commercial preparation by means of ionex chromatography and continuous free-flow electrophoresis.

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** Abbreviations: LVP, [8-lysine]-vasopressin; [^{3}H]-LVP, [^{3}H] [8-lysine]-vasopressin; BSA, bovine serum albumin; pIgG, porcine immunoglobulin; poly-Glu, poly-L-glutamic acid; ECDI, 1-ethyl-3-(3'-dimethyl-aminopropyl) carbodiimide methoiodide; DCDI, dicyclohexylcarbodiimide.

EXPERIMENTAL

The crude LVP preparation (activity 100 I.U./ml, pH 3—3·5) was a product of Léčiva, Prague, Czechoslovakia. [^{3}H]-LVP with a specific activity of 1·3 Ci/mmol was prepared and purified on a column of CM-Sephadex according to Carlsson and coworkers[3], on a column of Bio-Gel P-2 and by paper electrophoresis[4]. Purified [^{3}H]-LVP was used for experiments no later than 16—18 h after electrophoresis and elution. Amberlit IRC 50 was purchased from Koch-Light Lab. Ltd., Colnbrook-Bucks, England, BSA from Sevac, Michalany, Czechoslovakia, pIgG was a gift from Dr F. Franěk of this Institute. Poly-Glu was prepared by the polymeration of γ-benzyl-N-carboxy--L-glutamate anhydride which had been synthetized from γ-benzyl-L-glutamate[5]. γ-Benzyl-L-glutamate was kindly provided by Dr K. Bláha of this Institute. The protecting group was removed by the action of hydrogen bromide[6] under anhydrous conditions. Components with low molecular weight were removed by chromatography on Sephadex G-75. Polymers with molecular weights of 40000—50000 were used in the experiments. ECDI and DCDI were products of Fluka, AG, Buchs, Switzerland, 2-ethyl-5-phenylisoxazolium-3′-sulphonate (Woodward's reagent) was purchased from Aldrich, Milwaukee, U.S.A.

Methods

Ionex chromatography of the crude LVP preparation was performed according to Zaoral[7] on a 2·5 × 30 cm column of Amberlit IRC 50 (H^+ cycle) in equilibrium with 0·25% CH_3COOH. After adsorption of the total amount (c. 100—150 ml), the column was washed with 100—150 ml of 0·25% acetic acid. Elution was performed with 50% acetic acid at a rate of 1 ml/min and checked by determining the sodium content by means of a flame photometer, the pressor activity in biological assays, and absorption at 280 nm in each fraction. Fractions containing biologically active material were combined and freeze-dried.

High-voltage continuous free-flow electrophoresis was carried out in a Hannig type apparatus as modified by Prusík[8]. The apparatus had a planar slit measuring 500 × 500 × 0·6 mm with an effective length of 440 mm from the entrance of the sample into the slit to the end of the slit at the collector. Separation took place in 0·5M acetic acid, potential gradient 60 V cm^{-1}, exit temperature of cooling air —2°C. The electrodes were washed with 1M acetic acid. A 4·5% solution of the sample was applied at a distance of 85 mm from the anode membrane. Passage of the sample through the electric field took 60 min. The absorption at 280 nm and pressor activity were determined in individual 5 ml fractions. The fractions corresponding to the individual peaks of absorption were combined and freeze-dried. The samples were then analysed by paper electrophoresis, their amino acid composition and biological activity were determined.

The pressor activity was assayed on pithed male rats of the Wistar strain, weighing 180—220 g, according to the method of Sawyer[9] as modified by Krejčí and coworkers[10]. The changes in blood pressure were registered using a Statham A 23 tensometer. The specific activity (pressor potency) of the individual fractions was determined by the four-point test using [8-arginine]-vasopressin or synthetic LVP as standards.

Measurement of radioactivity. 5—10 ml of scintillation solution according to Bray[11] were added to the samples (50—100 μl). Scintillation was measured by the Packard-Tri-Carb Scintillation Spectrometer, Model 3375. The effectivity of the individual measurements was in the range of 20—25%.

Formation of the peptide bond by the carbodiimide reaction[12]. LVP together with [^{3}H]-LVP were added to an aqueous or saline (0·01—1M-NaCl) solution of the carrier (BSA, pIgG), and the

pH of the mixture was adjusted to the value required (5—7·5) using Na-phosphate buffer, HCl or NaOH. The reaction was started by the addition of an aqueous solution of ECDI and the volume of the reaction mixture was made up to 1·5 ml. The mixture was stirred gently for 2—24 h at room temperature. DCDI in dimethylformamide solution was used for binding to poly-Glu.

Formation of the peptide bond by Woodward's reagent[13]. Triethylamine was added to the mixture of carrier and labelled and unlabelled hormone in order to adjust the pH value to 8—12·4. The reaction was started by the addition of an aqueous or dimethylformamide solution of Woodward's reagent. The reaction mixture (volume 1·5 ml) was stirred gently for 24 h at 0°C.

Removal of free hormone from the hormone-carrier complex. Two methods were used in the case of protein carriers. After the termination of the reaction the sample was dialyzed against water or saline for 48 h with repeated exchanges of the dialysation medium. Samples of the dialysation medium were assayed for the presence of radioactivity. The second method consisted of the separation of the free from the bound hormone by gel filtration on a 1 × 48 cm column of Sephadex G-25. The radioactivity of the elution peaks containing protein and free hormone was compared. In the case of poly-Glu the reaction was terminated by diluting the reaction mixture with water which led to the precipitation of the conjugate. The mixture was centrifuged, the sediment washed and centrifuged. Radioactivity was determined in the sediment and in the supernatant. The amount of hormone bound to poly-Glu was also determined by amino acid analysis of the conjugate.

RESULTS

Isolation of [8-*lysine*]-*vasopressin.* Ionex chromatography on a column of Amberlit IRC 50 removed not only inorganic salts but also peptide material (peak *I* in Fig. 1) with insignificant biological activity (0·5—1·7 I.U./mg). The fractions com-

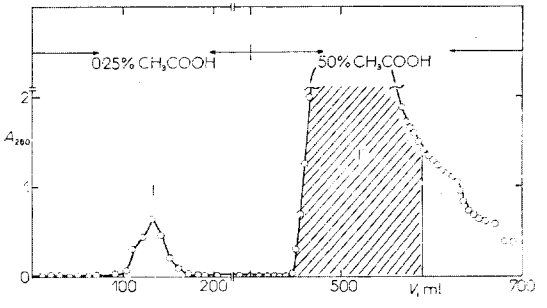

FIG. 1

Ionex Chromatography of the Crude [8-Lysine]-vasopressin Preparation on a Column of Amberlit IRC 50

Elution was performed in two steps *I* the column was washed with 400 ml of 0·25% CH_3COOH (salts were present between 60 and 130 ml of eluate), *II* by 300 ml of 50% CH_3COOH. Abscissa: elution volume in ml, ordinate: absorption at 280 nm. The part of the eluate containing biologically active material (cross hatching) was freeze-dried and subjected to further purification. For details, see text.

prising peak *II*, which contained the compound with pressor activity, were combined and freeze-dried. The biological activity of the residue was 80–120 I.U./mg.

Continuous free-flow electrophoresis of the evaporation residue of peak *II* resulted in further separation (Fig. 2). Biological activity was found in peak *I*. The amino acid analysis of the peptide material contained in peak *I* (composition: Lys 1·00, Asp 1·12, Glu 1·12, Pro 0·98, Gly 1·02, Cys 1·92, Tyr 0·96, Phe 1·04) showed the exclusive presence of LVP. The biological activity of LVP obtained by this isolation procedure was 240–260 I.U./mg. The amino acid analysis of the peptide material from the other peaks proved the presence of hormone fragments; for example peak *II* contained the pentapeptide composed of Cys, Tyr, Phe, Gln, Asn together with traces of LVP, and peak *IV* all amino acids of the hormone with Gln and Asn in double the amount of the other amino acids.

The procedure yielded 50–60% of LVP of the above-mentioned biological activity (with the exception of one case, in which bacterial contamination occurred resulting in endopeptidase cleavage of the hormone between lysine and glycineamide, *i.e.* trypsin-like cleavage).

Formation of the peptide bond between LVP *and the carrier.* Table I presents the results of the binding of LVP to BSA and pIgG by means of soluble carbodiimide. The concentration of LVP was 30–100 μM and carbodiimide was added in 10–20 times higher concentrations. A higher amount of carbodiimide (a 100–150 molar excess) added to the given concentration of hormone and BSA did not increase the binding of the hormone to the carrier. When pIgG was used as carrier, the addition of larger amounts of carbodiimide resulted in the formation of insoluble precipitates within several minutes. Apparently, side reactions took place,

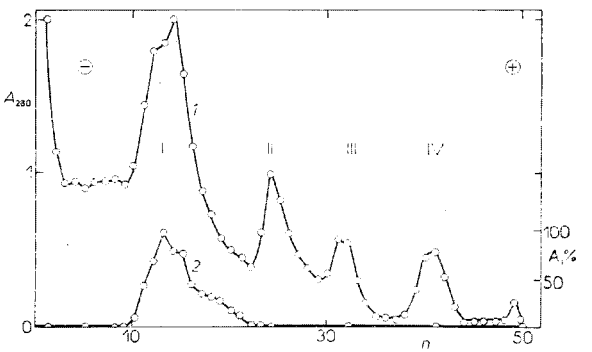

FIG. 2

Free-Flow Electrophoresis of [8-Lysine]-vasopressin

1 Absorption at 280 nm; *2* biological activity (the pressor activity of the most active fraction is given as 100%); *n* number of fractions. For details, see text.

bonds were formed between the individual functional groups of the immunoglobulin, and the resultant immunoglobulin polymers had cross-linked structure. The maximum of covalent bonds between the hormone and carrier was achieved after 2–4 h; further additions of carbodiimide did not affect the amount of hormone bound. No influence of salts on the binding of the hormone to the carrier was observed in the range of 0·01 – 1·00M-NaCl.

The reaction between poly-Glu and LVP was performed under similar reaction conditions to those mentioned above. The results are given in Table I. Increasing the amount of carbodiimide in the reaction to a 500 fold molar excess of the hormone led to higher binding of LVP to the synthetic carrier by one order of magnitude.

Table II presents result of the binding of the hormone to BSA and pIgG by means of Woodward's reagent. The reagent was added in a 10 – 15 molar excess to LVP, the concentration of which was 30 – 100 μM. As the pH value increased (8 – 12·4) the binding of LVP to the carrier rose by an order of magnitude.

If Woodward's reagent and triethyl amine are used in the same molar excess as DCDI, the percentage of the binding of LVP to poly-Glu is approximately the same. Both an increase of the concentration of Woodward's reagent and a rise in the pH value cause higher binding of LVP to poly-Glu. Amino acid analysis of the conjugates of LVP and poly-Glu supported the results obtained in experiments with the [^{3}H]-LVP marker.

DISCUSSION

Inactive peptide material was removed from the crude preparation of LVP during desalting on a column of Amberlit IRC-50. The biologically active material still

TABLE I

Binding of [8-Lysine]-vasopressin to Bovine Serum Albumin, Porcine Immunoglobulin and Poly-L-glutamic Acid by the Carbodiimide Reaction

Weight ratio BSA : LVP	Binding[a]	Weight ratio pIgG : LVP	Binding[a]	Weight ratio poly-Glu : : LVP	Binding[a]
500 : 1	0·014	250 : 1	0·008	200 : 1	0·05
50 : 1	0·089	50 : 1	0·056	100 : 1	0·33
20 : 1	0·100	25 : 1	0·076	40 : 1	1·05
10 : 1	0·180	10 : 1	0·370	20 : 1	1·80
5 : 1	0·315	5 : 1	0·450	10 : 1	2·10
2 : 1	0·860	2 : 1	1·410		
1 : 2	1·260				

[a] Expressed in mol of hormone per mol of carrier.

contained certain peptide components which could be removed by high-voltage free-flow electrophoresis. Amino-acid analysis, paper electrophoresis and biological assays proved that it is possible to separate LVP from the other products its synthesis by the above-mentioned procedure. As in earlier experiments with natural [8--arginine]-vasopressin[14], synthetic kallidin, and bradykinin analogues[15], free-flow electrophoresis was successfully used for the isolation of LVP. The LVP preparation obtained in this way was used for studying the formation of peptide bonds between the carboxyl groups of carriers and the amino groups of LVP.

Three compounds with different contents of carboxyl groups were tested as carriers, the number of carboxyl groups being the limiting factor in the given condensation reactions. BSA and pIgG contain such numbers of carboxyl groups that it would theoretically be possible to bind 105 and 237 molecules of haptene, respectively, to one molecule of carrier[16]. However, the binding is severely limited by the reactivity of the individual carboxyl groups and by the presence of other functional groups in the carrier molecule (possible formation of cross links between carrier molecules, as indicated by the fact that precipitation occurred in experiments with immunoglobulin). Poly-Glu has approximately 315 carboxyl groups per molecule. The conditions under which the reaction is performed and the properties of the haptene bound are other factors that influence binding.

A comparison of the amount of LVP bound to these carriers in the case when carbodiimide was used as a condensation reagent shows that the greatest amount of hormone was bound to poly-Glu; at a 20 : 1 weight ratio of carrier to hormone, 25 times more hormone was bound to poly-Glu than to protein carriers. The maximum amount bound to protein carriers was 1·26 mol of LVP/mol BSA and 1·41 mol of LVP/mol pIgG; thus, every 50th molecule of hormone reacted.

When we compare the reaction using Woodward's reagent with that applying carbodiimide, we find that carbodiimide is 4–10 times more effective in binding the

TABLE II

Binding of [8-Lysine]-vasopressin to Bovine Serum Albumin and Porcine Immunoglobulin by Woodward's Reagent

Weight ratio BSA : LVP	Binding[a]	Weight ratio pIgG : LVP	Binding[a]
50 : 1	0·006	50 : 1	0·040
20 : 1	0·009	25 : 1	0·050
10 : 1	0·016	10 : 1	0·130
5 : 1	0·033	5 : 1	0·295

[a] Expressed in mol of hormone per mol of carrier.

hormone to protein carriers. This is probably due to the lower specificity of the carbodiimide reaction[17]. The binding of LVP to poly-Glu was approximately the same, regardless of the method used.

Data concerning the amount of LVP bound covalently to various carriers in conjugates used for immunisation have as yet been very scarce. Our results on the binding of LVP to BSA agree well with data presented by other authors[18,19]. The binding to IgG could be compared only with the results of one work (Wen-hsien Wu and Rockey[20]) in which 5 times higher values are presented. The difference might be caused by higher concentrations of the reaction components. Data concerning the content of LVP in conjugates with poly-Glu are also reported[18]. However, the results cannot be compared with ours because the authors do not state the molecular weight of polyglutamic acid used, nor do they use the same concentrations of reaction components.

The binding of LVP by means of Woodward's reagent increased when the pH value rose, which can be explained by the course of the ionisation of the LVP molecule (this explanation is indirectly documented by the observation that a similar shift of the pH value did not result in increased binding of oxytocin[21]). The dependence of binding on pH does not have great practical significance due to the fact that neurohypophysial hormones are unstable in alkaline media.

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