Isolation and characterization of phosphorylated bovine prolactin

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Quantitative isolation of bovine prolactin was accomplished by immunoaffinity chromatography using clonal antibody as the stationary ligand. The phosphorylated and non-phosphorylated (native) prolactins contained in the immunopurified preparations were separated by chromatofocusing. Isolates from individual pituitaries revealed that phosphorylated prolactin represented between 20 and 80% of the total prolactin. The stoichiometry of phosphate in phosphorylated prolactin was 1.4:1 when determined by amino acid analysis after preparation of the *S*-ethylcysteine derivative. One major phosphorylation site, serine-90, and two minor sites, serine-26 and -34, were determined by mapping and sequencing studies. Serine-90 was conserved in prolactins, growth hormones and placental lactogens. Serine-26 and -34 were conserved in prolactins, but were not found in growth hormones or placental lactogens. Absorption spectroscopy of the aromatic amino acid residues indicated that phosphorylation of prolactin was associated with a unique structural conformation.

INTRODUCTION

Hormone variants are produced by several mechanisms, which include genetic mechanisms (the expression of heterologous genes [1,2] and alternative patterns of RNA splicing [3,4]) or post-translational mechanisms (including alternative cleavage sites of the prohormone [5–7], deamination [8], glycosylation [8] and phosphorylation [9,10]). Isolation and determination of the structures of these hormone variants are necessary prerequisites to understanding the relationship of structure and function.

Post-translational modifications of many proteins provide a means to regulate diverse biological actions. The hypothesis that post-translational modification is a mechanism that creates several structurally and functionally unique hormones from one gene product is a relatively recent concept in endocrinology. The post-translational modification of pro-opiomelanocortin by cleavage and glycosylation to create several hormones with unique functional characteristics is one well-documented example [11]. This hypothesis has yet to be examined thoroughly for other hormone families by isolation and characterization of hormone variants and comparison of structural features with biological activities.

The existence of functional prolactin variants has been demonstrated most frequently by differing ratios of biological and immunological activities among preparations [12–14]. Structural variants of prolactin have been reported to be produced by polymerization [15–17], deamination [18], glycosylation [19–21] and phosphorylation [22–25]. Synthesis of phosphorylated prolactin was identified in rat pituitary cultures by Walker and colleagues [22]. Kinase reactions *in vitro* identified rat prolactin to be a substrate for the catalytic subunit of protein kinase A, protein kinase C, protease-activated kinase I and casein kinase I [22]. The phosphorylation sites for these kinases have not been determined. One major and two minor more acidic isoforms of phosphorylated rat prolactin were detected by two-dimensional gel electrophoresis [23]. The phosphorylated isoform, but not the native one, has a unique biological function, suppressing prolactin secretion from GH3 cells [24]. We reported the synthesis and secretion of phosphorylated prolactin from bovine pituitary explants [25].

The relationship between specific structural features of phosphorylated prolactin and either biological or immunological properties is not understood. No results have been obtained on the regulation of phosphorylated prolactin production. A difficulty in these efforts is that no procedure for the isolation of phosphorylated prolactin variants has been described. We report the isolation of phosphorylated and non-phosphorylated (native) bovine pituitary prolactin and the determination of the sites of phosphorylation. In addition, we observe that the behaviours of native and phosphorylated prolactins during chromatofocusing chromatography and absorption spectroscopy are not explained solely by the presence of phosphate, suggesting that conformational changes may be induced by phosphorylation in a manner similar to many post-translationally regulated enzymes.

EXPERIMENTAL

Materials

Bovine prolactin (U.S.D.A. Animal Hormone Program, biological grade) was provided through the National Hormone and Pituitary Program (N.H.P.P.). Bovine pituitaries were obtained from a local meat packer. Retired-breeder female Balb/c mice were purchased from Harlan Sprague–Dawley (Indianapolis, IN, U.S.A.) and maintained in a U.S.D.A.-inspected facility under the procedures of an approved protocol. Protein-A affinity columns were purchased from ChromatoChem Inc. (Missoula, MT, U.S.A.). Matrix for immunoaffinity chromatography (AminoLink coupling gel, catalogue no. 20382), standards and reagents for amino acid analysis and tosylphenylalanylchloromethane ('TPCK')-treated trypsin immobilized to agarose beads (catalogue no. 20230) were purchased from Pierce Chemical Co. (Rockford, IL, U.S.A.). Supplies for chromatofocusing, including Mono P columns and Polybuffer 74 ampholine mixture, were purchased from Pharmacia–LKB Biotechnology (Piscataway, NJ, U.S.A.). Carrier-free [^{32}P]P_i was obtained from ICN Biomedicals (Costa Mesa, CA, U.S.A.). Dulbecco's

Abbreviations used: N.H.P.P., National Hormone and Pituitary Program; PVDF, poly(vinylidene fluoride); TFA, trifluoroacetic acid.

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Modified Eagle's medium with 10 μg/l phosphate was prepared by Gibco (Grand Island, NY, U.S.A.). Alkaline phosphatase from calf intestine (catalogue no. ALPI11G) was purchased from Biozyme Laboratories International (San Diego, CA, U.S.A.). *S*-Ethyl-L-cysteine was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other reagents were of the highest purity available and were obtained from various commercial sources.

Incorporation of [^{32}P]P_i into bovine prolactin *in vivo*

Anterior pituitaries were cut into explants with an estimated size of 1 mm^3 and transferred to 10 ml screw-cap Erlenmeyer flasks containing 3 ml of low-phosphate Dulbecco's Modified Eagle's medium (10 μg of phosphate/l) and 1 mCi of [^{32}P]P_i/ml. The explants was incubated for 3 h in a reciprocating water bath at 37 °C and washed three times with extraction buffer (10 mM Tris, pH 7.0, 0.3 M sucrose, 5 mM EDTA, 5 mM EGTA, 1 mM phenylmethanesulphonyl fluoride, 10 μg of aprotinin/ml and 0.5% Triton X-100).

Preparation of anterior-pituitary extracts

The washed explants or fresh individual anterior pituitaries were homogenized in 5 ml of extraction buffer with a PT-10 Polytron (Brinkman Instruments) for 1 min at setting 6. The homogenate was transferred to a centrifuge bottle with an additional 5 ml of homogenization buffer and subjected to centrifugation at 145000 *g* for 1 h at 4 °C. The supernatant was retained and stored at −20 °C.

Preparation of anti-(bovine prolactin) clonal antibody and affinity resins

Bovine prolactin (N.H.P.P. preparation) was injected subcutaneously (100 μg per dose) into Balb/c mice until a titre was detected by e.l.i.s.a. Spleen cells were prepared, fused and cloned by the method of Oi and Hertzenberg [26] in The Monoclonal Antibody Laboratory at this institution. Clones were selected on the basis of successful growth in culture and in the peritoneal cavity of mice, production of IgG, and the affinity of antibody and antigen. One clone was selected, and 2×10^6 cells were injected into pristane-treated female Balb/c mice. Ascites fluid was harvested, and the lipid and cells were removed by centrifugation.

Mouse IgG was purified in a single step by Protein-A affinity chromatography. A 20 ml portion of ascites was diluted with an equal volume of PBS (10 mM sodium phosphate buffer, pH 7.5, 150 mM NaCl) and applied to a 21.6 mm × 250 mm column (capacity 1.1 g of human IgG) at a flow rate of 10 ml/min. The column was washed with PBS at a flow rate of 40 ml/min until the A_{280} returned to baseline, whereupon the IgG was eluted with 2% acetic acid containing 150 mM NaCl. The eluate containing IgG was neutralized with NaOH, dialysed against 5 mM NH_4HCO_3 and subsequently freeze-dried. Yields of 100 mg of IgG could be purified to greater than 95% in one chromatographic step. The recovery of IgG by this method was greater than 95%.

Affinity resin (10 ml of settled resin/column) was washed three times with coupling buffer (100 mM sodium phosphate, pH 7.0). IgG (100 mg) was resuspended in 10 ml of coupling buffer and added to the resin cake. The coupling reaction was initiated with the addition of 2 ml of 1 M sodium cyanoborohydride and was run at room temperature for 2 h on a rotary platform shaker, followed by 2 h without agitation. The gel was washed with 1 M Tris/HCl, pH 7.4, and incubated in this buffer with the addition of 2 ml of 1 M sodium cyanoborohydride overnight at 4 °C to block the remaining active sites. The product was transferred to a glass column and washed extensively in PBS. Measurement of the antibody in solution before and after cross-linking to the resin revealed that between 63 and 75% of the 100 mg of IgG was bound to the resin, providing calculated antibody densities between 21 and 30 nmol/ml and a maximum column capacity between 4.6 and 6.6 mg of bovine prolactin. The columns were stored at 4 °C in PBS (pH 7.45) containing 0.02% NaN_3.

Immunoaffinity chromatography

Affinity chromatography was performed at room temperature in PBS (pH 7.45) at a flow rate of 1 ml/min. The extract from one pituitary (10 ml) was diluted 1:1 with PBS and applied to the affinity column. The sample was recirculated over the resin for 3 h. The resin was washed for 2.5–3 h with PBS. Finally, the retained protein was eluted with 0.1 M glycine, pH 2.8. The solution was returned to a neutral pH with NaOH and dialysed at 4 °C against 5 mM NH_4HCO_3, adjusted to pH 7.5 with acetic acid. The protein was dried and stored at −20 °C.

Separation of native and phosphorylated bovine prolactin by chromatofocusing

Immunopurified bovine prolactin was solubilized in less than 2 ml of buffer (25 mM Bistris, pH 7.1, with iminodiacetic acid). The sample was applied to a Mono P anion-exchange column previously equilibrated with the buffer and washed for 10 min at 1 ml/min by a high-pressure chromatograph (Waters Division, Millipore Corp., Bedford, MA, U.S.A.). A pH gradient from 7.0 to 4.0 was generated by pumping 10% Polybuffer 74 (pH 4 with iminodiacetic acid) through the column for 45 min at 1 ml/min, followed by a 10 min wash with PBS to decrease the amount of ampholytes remaining on the column. The retained prolactin was eluted by a 2 ml wash with 70% acetic acid. Proteins, detected by their A_{280}, were collected and precipitated by $(NH_4)_2SO_4$ (80% satn.), followed by dialysis against 5 mM NH_4HCO_3 (pH 7.5 with acetic acid) and freeze-drying.

Analytical methods

Prolactin preparations were analysed by amino acid analysis [27] and SDS/PAGE (12% acrylamide gel) [28].

Phosphate stoichiometry

The stoichiometry of phosphate and prolactin were determined by measuring the ratio of a stable phosphoserine derivative (*S*-ethylcysteine) and the non-phosphorylated serines by amino acid analysis. This approach will not be influenced by the presence of free phosphate in the sample.

Cysteines were reduced and blocked before the amino acid analysis, as this residue was eluted from the amino acid chromatogram in the region of *S*-ethylcysteine. Cysteines were reduced with 2-mercaptoethanol and blocked with 4-vinylpyridine [29], and the prolactin was recovered by reverse-phase chromatography [3.9 mm × 300 mm Delta-Pak C_{18} column with a 300 nm mean pore size; Waters Division, Millipore Corp.; 25 min 0–100% acetonitrile gradient on a water/0.1% trifluoroacetic acid (TFA) background].

The phosphoserines were converted into a stable derivative, *S*-ethylcysteine, by the method of Meyer et al. [30]. Phosphate was cleaved by a β-elimination reaction (0.1 M NaOH/1 mM $CaCl_2$ for 2 h at 40 °C [31]), followed by reverse-phase purification, drying and subsequent reduction of the dephosphorylated residues with thioethane (0.1 M, overnight at room temperature). The product was purified by reverse-phase chromatography, dried, and prepared for amino acid analysis [27].

Stoichiometry was calculated from the molar ratios of the *S*-ethylcysteine and serine (15 mol of serine per mol of prolactin minus one mol of phosphoserine, corrected for 90% recovery from hydrolysis).

Peptide mapping and sequence determination

Peptide maps of prolactin preparations were prepared by incubating prolactin at a concentration of 1 mg/ml in 100 mM $(NH_4)_2CO_3$/0.02% NaN_3 with a ratio of 1:25 of trypsin/agarose to prolactin (> 9 units of enzyme/mg of prolactin). The reaction was run for 24 h at 37 °C, and 600 μl of reaction mixture was injected on to a reverse-phase column (3.9 mm × 300 mm; Delta-Pak C_{18}) and eluted with a 0–60% acetonitrile gradient on a 0.1% TFA/water background over 60 min, with 1 ml fractions being collected.

Eluted peptides were identified by A_{214}, and all fractions were dried. Phosphopeptides were initially identified by performing mapping experiments with bovine prolactin preparations that had been labelled *in vivo* with [^{32}P]P_i. These peptides were identified by reconstituting the dried samples in 100 μl of buffer (10 mM Tris, pH 7.5, 150 mM NaCl with 0.01% NaN_3 and 1 μM $CaCl_2$) and placing 80 μl on to poly(vinylidene difluoride) (PVDF) membranes (Immobilon P, Millipore Corp.). [^{32}P]P_i and ^{32}P-labelled prolactin were also applied to additional wells in the manifold to determine if the PVDF matrix would retain P_i or organic phosphate. The membranes were washed with water, dried and exposed to film. Fractions containing phosphopeptides were identified from the film. This radiographic procedure was more sensitive than scintillation counting and eliminated peaks of free [^{32}P]P_i and [^{32}P]ATP, as they were not retained by the PVDF matrix. Peptides in subsequent mapping and sequencing experiments were selected by their retention times, applied to the PVDF matrix and extensively washed with water before drying.

Phosphate is lost from phosphoserine during sequencing by either a β-elimination reaction, leaving a dehydroalanyl residue, or acid hydrolysis. In preliminary sequencing experiments that identified the phosphopeptide, little or no phosphoserine survived sequencing and the recovery of dehydroalanine was low. Thus derivative formation of phosphoserine was necessary before the identification of phosphorylated residues.

Phosphopeptides from immunopurified bovine prolactin were isolated by mapping, and stable phosphoserine derivatives were made by the method of Meyer et al. [30] to facilitate the identification of the phosphate-containing residues during sequence analysis. None of the peptides with potential sites of phosphorylation contained cysteine residues; therefore reduction and alkylation were not performed before β-elimination reactions. Simultaneous β-elimination of the phosphate (0.1 M NaOH/1 mM $CaCl_2$, 40 °C for 2 h) and reduction of the dehydroalanine with thioethane (0.1 M) produced *S*-ethylcysteinyl derivatives from phosphoseryl residues. The peptide derivatives were purified by reverse-phase chromatography, dried, applied to a PVDF matrix and washed with water.

Fractions of interest were cut from the membrane and the peptide sequence was determined by a gas-phase method using an Applied Biosystems model 475A Protein Sequencer (Foster City, CA, U.S.A.), with the amino acids identified as their phenylthiohydantoin derivatives [32].

Absorption spectroscopy

Absorption spectroscopy was performed on a Varian model DMS 300 double-beam spectrophotometer between 240 and 340 nm. Samples of native, phosphorylated and N.H.P.P. bovine prolactins were placed in 10 mM sodium phosphate buffer, pH 7.4, with 150 mM NaCl, at a protein concentration between 200 and 250 μg/ml.

RESULTS

Prolactin was quantitatively isolated from extracts of the bovine pituitary by immunoaffinity chromatography (Figures 1 and 2) with yields between 1 and 5 mg per pituitary. In multiple experiments all 22 kDa bovine prolactin was removed from the pituitary homogenate (Figure 2, lanes 4 and 5) as determined by SDS/PAGE of the column flow-through fraction. The immunopurified prolactin was greater than 95% homogeneous, as judged by SDS/PAGE (Figure 2, lanes 6 and 7). The most frequent contaminants were proteins with molecular masses of approx. 43 and 65 kDa (results not shown). The affinity columns were durable, with functional lives of approx. 6 months and capacity to perform satisfactorily in more than 25 purifications. When pituitary proteins were metabolically labelled with [^{32}P]P_i and immunopurified, autoradiography of SDS/PAGE gels revealed prolactin to be a phosphoprotein (Figure 3, lane 1), as we have previously reported [25]. Amino acid analysis of immunopurified bovine prolactin revealed a composition similar to that determined from nucleic acid sequence data [33].

Immunopurified bovine prolactins from individual pituitaries were separated into two major fractions by chromatofocusing (Figure 4). The first fraction was eluted as two or three peaks

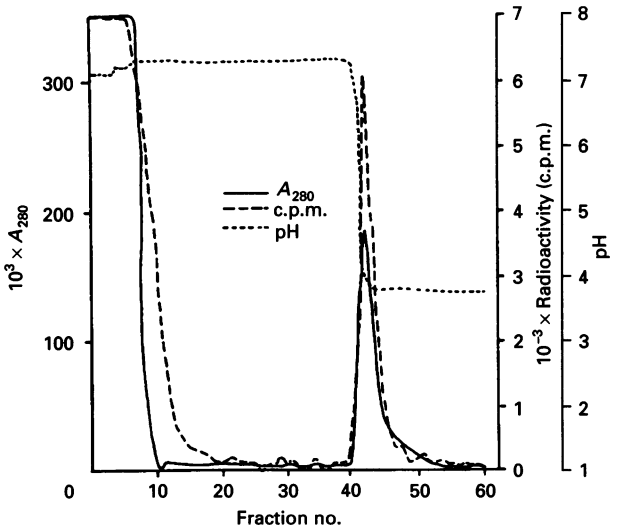

Figure 1 Immunoaffinity purification of bovine prolactin

Extract from a single [^{32}P]P_i-labelled bovine anterior pituitary was applied and recirculated at 1 ml/min on to a 10 ml antibovine IgG affinity column. At zero time the column was washed with PBS, pH 7.45. When the A_{280} had returned to baseline, the column was eluted with 0.1 M glycine (pH 2.8); 1 ml fractions were collected and monitored for pH, A_{280} and radioactivity (c.p.m./50 μl).

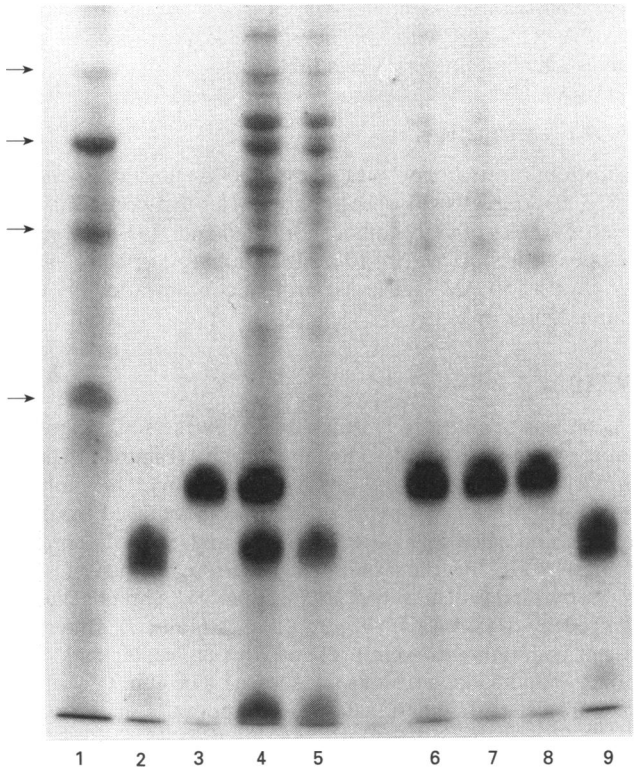

Figure 2 Coomassie-Blue-stained SDS-containing 12%-acrylamide gel prepared with 2-mercaptoethanol of immunopurified bovine prolactin

Lane 1, molecular-mass markers (arrows: 95, 66, 43 and 28 kDa); lanes 2 and 9, bovine growth hormone standard; lanes 3 and 8, bovine prolactin standard; lane 4, pituitary extract; lane 5, material not retained by affinity column; lanes 6 and 7, immunopurified bovine prolactin from two pituitary preparations.

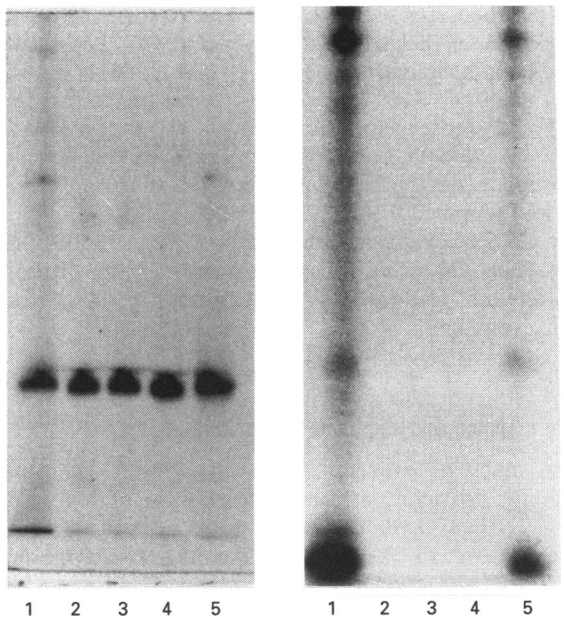

Figure 3 SDS-containing 12%-acrylamide gel prepared with 2-mercaptoethanol, and autoradiograph of native and phosphorylated bovine prolactins separated by chromatofocusing

Left panel, Coomassie-Blue-stained gel; right panel, autoradiograph. Lane 1, immunopurified bovine prolactin; lanes 2, 3 and 4, chromatofocusing gradient peaks at 30, 32 and 40 min respectively; lane 5, acid wash peak at 65 min.

between pH 5.9 and 5.7, and the second fraction was eluted in the acid wash at pH 2.7. Alternatively, the second fraction was eluted with methanol. Chromatofocusing of several immunopurified bovine prolactin preparations showed that pituitaries contained variable ratios of these two major prolactin variants and that the phosphorylated prolactin represents between 20 and 80 % of the immunopurified prolactin. SDS/PAGE and autoradiography of the proteins eluted at pH 5.9–5.7 (Figure 3, left panel, lanes 2–4) and 2.7 (Figure 3, left panel, lane 5) demonstrate that all peaks have similar molecular masses and co-migrate with the immunopurified bovine prolactin. Only the prolactin eluted at pH 2.7 is a phosphoprotein, as demonstrated by the co-migration of [^{32}P]P_i (Figure 3, right panel, lane 5).

The amino acid compositions and peptide maps of native and phosphorylated bovine prolactins were similar to those of N.H.P.P. bovine prolactin (results not shown).

We observed a significant loss of the prolactin-associated phosphate with exposure to extremes of pH. For example, chromatography of [^{32}P]phosphorylated prolactin on reverse-phase chromatography with TFA serving as an ion-pairing agent and subsequent freeze-drying resulted in phosphate being lost from the protein in a time-dependent manner. Equivalent losses of organic phosphate were not observed at neutral pH. Neutralization of extremely acidic or basic conditions was performed and resulted in increased yields of phosphoprotein.

Chromatofocusing of N.H.P.P. bovine prolactin (USDA-bPRL-B1) partitioned the protein into a large double peak at pH 5.7–5.9 and a very small amount of phosphorylated prolactin (pH 2.7) (Figure 5). These results indicated that this prolactin preparation, commonly used for bioassays, was composed primarily of non-phosphorylated native prolactin.

Studies of the stoichiometry of phosphate in isolates of phosphorylated prolactin by derivative formation with thioethane yielded a ratio of 1.4 mol of *S*-ethylcysteine/mol of prolactin (*S*-ethylcysteine standard measured by dry weight).

Immunopurified bovine prolactin isolated from [^{32}P]P_i-labelling studies of pituitary explants was mapped by reverse-phase chromatography after digestion with trypsin (Figure 6). Dot-blotting of the isolated peptides followed by autoradiography (Figure 7) indicated that peptides eluted at approx. 42 min were radioactive. Free [^{32}P]P_i was not retained by the PVDF membrane, whereas [^{32}P]phosphorylated prolactin was retained. Treatment of [^{32}P]phosphorylated prolactin with alkaline phosphatase before trypsin treatment eliminated the radioactivity associated with fraction 42. Peptide retention times of non-phosphorylated, phosphorylated and alkaline phosphatase-treated preparations of bovine prolactin were similar, indicating that the presence or absence of phosphate on the peptides eluted at 42 min did not significantly affect retention on the C_{18} packing in the 0.1 % TFA/water solvent (pH 2.5). Perhaps the phosphate is protonated at this low pH, minimizing the effect of phosphate charge on the retention time. The retention-time data were supported by sequencing data, where fraction 42 contained identical sequences of peptides cleaved from either phosphorylated or dephosphorylated preparations of bovine prolactin.

Preliminary work revealed that phosphopeptides were labile to the conditions encountered during sequencing, and phosphate-containing sequence products were not eluted from the PVDF matrix: therefore, measuring the radioactivity eluted with the

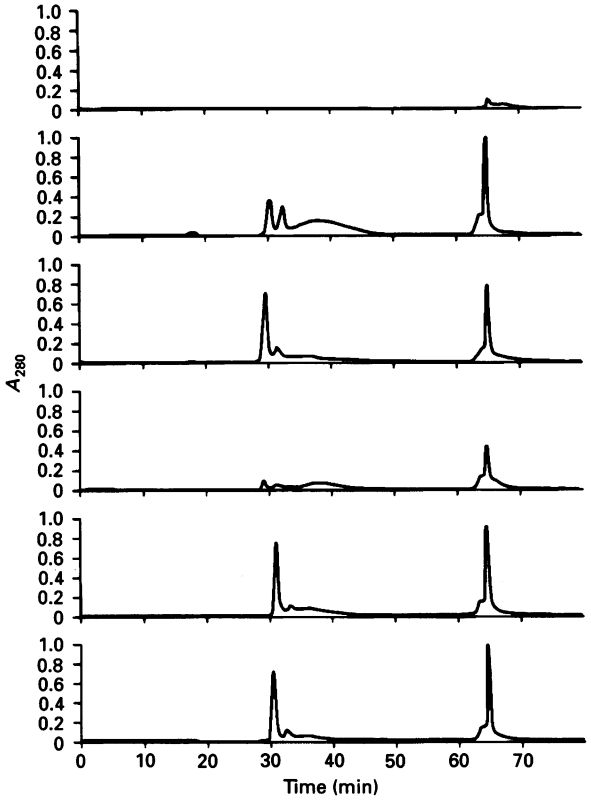

Figure 4 Separation of native and phosphorylated bovine prolactins by chromatofocusing

Top panel, background chromatograph; bottom five panels, separation of native and phosphorylated bovine prolactins from immunopurified preparations from extracts of single pituitaries. The ampholyte gradient was begun at 5 min (pH 7.1) and was completed at 50 min (pH 4.0), the starting buffer was run from 50 to 60 min, and 2 ml of 70% acetic acid was injected at 60 min. The A_{280} of the eluate was monitored.

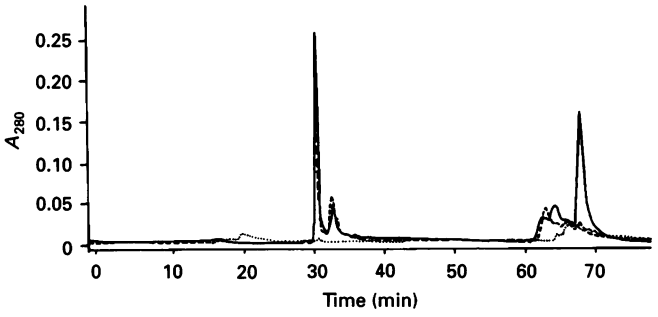

Figure 5 Chromatofocusing of the N.H.P.P. bovine prolactin (USDA-bPRL-B1)

Chromatofocusing was performed as described in Figure 4. ·····, Background; ——, immunopurified prolactin; ----, N.H.P.P. prolactin.

products from each Edman cycle provided no information. We observed no phosphoserine and low yields of dehydroalanine in sequencing experiments. Thus derivative formation before sequence analysis was required for the peptides present in fraction 42.

Sequence analysis of the fraction eluted at 42 min revealed two peptides. Conversion of the phosphoamino acids by β-elim-

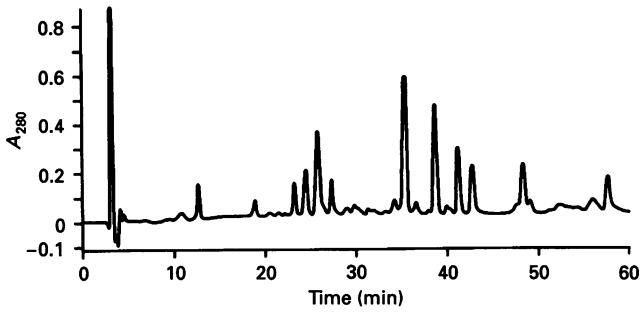

Figure 6 Tryptic-peptide map of immunopurified bovine ^{32}P-labelled prolactin

Immunopurified bovine prolactin was digested with trypsin–agarose, and the resulting peptides were separated by C_{18} reverse-phase chromatography with a 0–60% acetonitrile gradient on a 0.1% TFA/water background.

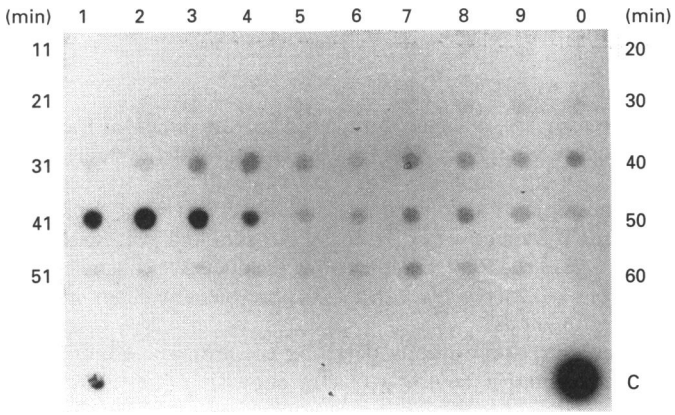

Figure 7 Autoradiographic detection of [^{32}P]phosphopeptides from tryptic-peptide maps of immunopurified bovine prolactin by PVDF-matrix dot analysis

Fractions were collected each 1 min (Figure 6), dried, resuspended in a small volume and placed on PVDF matrix. Control dot for ^{32}P-labelled prolactin is in the lower right corner (C). Free [^{32}P]P_i was not retained on the matrix.

ination and reduction with thioethane, followed by re-purification by reverse-phase chromatography, separated the two peptides. Sequence analysis identified the peptides as residues 22–42 and residues 90–103. *S*-Ethylcysteine (S*) was recovered at positions 26, 34 and 90, identifying these residues as phosphorylation sites.

residues 22–42 AVMVS*HYIHDLSS*EMFNEFDK

residues 90–103 S*WNDPLYHLVTEVR

The presence of *S*-ethylcysteine in specific sequencing cycles identified serine residues as the sites of phosphorylation: the absence of thioethane-derived peaks in sequencing cycles identifying threonine, tyrosine or other serine residues suggested that these residues were not phosphorylated.

Simultaneous sequence analysis of peptides 22–42 and 90–103 isolated from an immunopurified preparation of bovine prolactin showed approx. 78% of the phosphate to be associated with serine-90, whereas serine-26 and -34 accounted for only 13% and 9% respectively. These values were calculated from the ratios of *S*-ethylcysteine to serine from sequencing cycles 1, 5 and 13. In each of these cycles, the mol of serine indicated the total

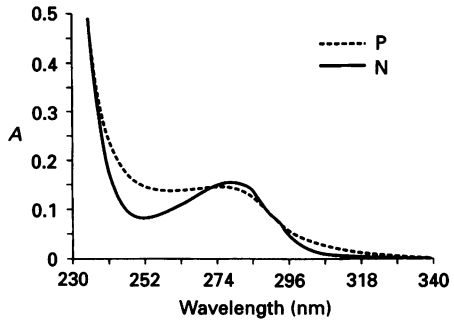

Figure 8 Absorption spectrophotometry of aromatic amino acid residues in native (N) and phosphorylated (P) bovine prolactin

Bovine prolactins were placed into 10 mM sodium phosphate (pH 7.0)/150 mM NaCl with or without guanidine hydrochloride. Spectra were obtained with a 0.5 nm slit width at a speed of 50 nm/min in a double-beam instrument with a buffer (blank). The maximum at 277 nm was measured for both proteins in the presence of guanidine hydrochloride; the ratio of these values was used to normalize the protein concentrations for the spectra shown in the Figure obtained without guanidine hydrochloride.

peptide recovery, whereas the *S*-ethylcysteine indicated the mol of phosphoserine. For each cycle, the mol of *S*-ethylcysteine were divided by the mol of serine to calculate the fraction of phosphoserine at that residue. These fractions were summed. Each initial fraction was divided by this sum and expressed as a percentage of the total phosphoserine residues. We calculate that a phosphorylated residue with a 2–3 % prevalence will be detected by these methods.

Absorption spectroscopy detecting the aromatic amino acid residues in native bovine prolactin showed a substantial absorption at 277 nm, with a tryptophan shoulder at 292 nm (Figure 8). In contrast, the absorption of phosphorylated bovine prolactin was decreased at 277 nm and had undergone a substantial blue shift. The 292 nm shoulder was absent from the spectra of phosphorylated bovine prolactin. The N.H.P.P. bovine prolactin absorption was similar to that of the native prolactin (result not shown).

DISCUSSION

Phosphorylated and non-phosphorylated bovine prolactin variants have been isolated from extracts of the bovine pituitary by a combination of quantitative immunoaffinity chromatography and chromatofocusing. During chromatofocusing, native bovine prolactin was eluted close to its calculated pI (5.80) between pH 5.7 and 5.9, and consisted of several subtypes. Phosphorylated bovine prolactin was eluted from the chromatofocusing resin at pH 2.7, and contained covalently bonded phosphate, which was susceptible to treatment with alkaline phosphatase and labile to extremes of pH. These data confirmed our previous observations that phosphorylated bovine prolactin was present and was synthesized in the anterior pituitary [25], and demonstrated that phosphorylated prolactin represents a major form of pituitary prolactin. The variation in the ratios of native and phosphorylated prolactins separated from individual pituitaries suggested that the kinase activity responsible for bovine prolactin phosphorylation may actively modulate the amounts of the native and phosphorylated variants which were subsequently available for secretion.

Elution of native and phosphorylated prolactins from the chromatofocusing column by a pH difference of several units was a unique observation. Addition of a single phosphate group to a protein should have decreased its pI by several tenths of a pH unit. We interpreted this observation to suggest that phosphorylation of bovine prolactin altered the conformation of the prolactin, providing substantially different surface-charge properties. Native and phosphorylated prolactins had similar amino acid compositions [25], molecular masses and peptide maps, suggesting that altered primary structure could not account for the properties of these prolactins during chromatofocusing. In addition, absorption-spectroscopic studies indicated that phosphorylation of bovine prolactin produced a blue shift in the absorption of the aromatic amino acid residues resulting from altered hydration, and demonstrating a change in conformation. Finally, preliminary studies using non-denaturing and non-reducing PAGE showed substantially different migration of isolated native and phosphorylated bovine prolactins.

Oetting and Walker [23] identified by two-dimensional gel electrophoresis one major phosphorylated prolactin and one or two minor species in rat pituitaries. The phosphorylation sites in bovine prolactin were conserved in the rat. The stoichiometry of 1.4 mol of phosphate/mol of prolactin and identification of one major and two minor phosphorylation sites suggested that phosphorylated prolactins were modified at serine-90 and that some of this population were also phosphorylated at either one or two minor sites. Thus phosphorylation at serine-90 is most likely to be responsible for the unique chromatographic and spectroscopic properties of phosphorylated prolactin. Resolution of this issue must wait until the subspecies of phosphorylated bovine prolactin can be either isolated or synthesized.

Examination of the aligned amino acid sequences of bovine [33], sheep [34], pig [35], horse [36], elephant [37], human [38], rat [39], mouse [40], whale [41], chicken [42] and turkey [43] prolactins revealed that serine-90 of bovine prolactin was conserved in each of these species (Figure 9). Alignment of bovine prolactin with bovine [44], sheep [45], pig [46], goat [47], horse [48], human [49], human variant-V [50], rat [51], mouse [40] and bullfrog [52] growth hormones and bovine [53] and human [54] placental lactogens demonstrated that the serine-90 residue was conserved in the protein family represented by these species. The serine-90

```
                20          30          40          80          90          100
                ^           ^           ^           ^           ^           ^
bPRL  ...RDLFDRAVMVSHYIHDLSSEMFNEFDKRY....LMSLILGLLRSWNDPLYHLVT...
oPRL  ...RDLFDRAVMVSHYIHNLSSEMFNEFDKRY....LMSLILGLLRSWNDPLYHLVT...
pPRL  ...RDLFDRAVILSHYIHNLSSEMFNEFDKRY....LLNLILRVLRSWNDPLYHLVT...
ePRL  ...RELFDRAVILSHYIHNLSSEMFNEFDKRY....LLNLILRVLKSWNDPLYHLVS...
elPRL...PDLFDRAVMLSHYIHSLSSDMFHEFNKQY....LMDLILGLLRSWNDPLDHLAS...
hPRL  ...RDLFDRAVVLSHYIHNLSSEMFSEFDKRY....FLSLIVSILRSWNEPLYHLVT...
rPRL  ...PELFDRVVMLSHYIHTLYTDMFIEFDKQY....LLNLILSLVHSWNDPLFQLIT...
mPRL  ...RELFDRVVILSHYIHTLYTDMFIEFDKQY....LVSLILGVLRSWNDPLYHLVT...
wPRL  ...RDLFDRAVILSHYIHNLSSEMFNEFDKRY....LVSLILGVLRSWNDPLYHLVT...
cPRL  ...GELFDRAVKLSHYIHYLSSEIFNEFDERY....LLNLVVGVLRSWNDPLIHLAS...
tPRL  ...GELFDRAVRLSHYIHFLSSEIFNEFDERY....LLNLILGVLRSWNDPLIHLAS...

bGH                                       ....LLRISLLLIQSWLGPLQFLSR...
oGH                                       ....LLRISLLLIQSWLGPLQFLSR...
pGH                                       ....LLRFSLLLIQSWLGPVQFLSR...
cGH                                       ....LLRISLLLIQSWLGPLQFLSR...
eGH                                       ....LLRFSLLLIQSWLGPVQLLSR...
hGH                                       ....LLRISLLLIQSWLEPVQFLRS...
hGHv                                      ....LLRISLLLIQSWLEPVQLLRS...
rGH                                       ....LLRFSLLLIQSWLGPVQFLSR...
mGH                                       ....LLRFSLLLIQSWLGPVQFLSR...
bfGH                                      ....LLRFSLTLLQSWMTPIQIVNR...

bPL                                       ....LLRLVISLLHSWDEPLHQAVT...
hPL                                       ....LLRISLLLIESWLEPVRFLRS...
```

Figure 9 Amino acid sequences of selected prolactins, growth hormones and placental lactogens surrounding the sites of phosphorylation

Prolactins: bovine (bPRL), sheep (oPRL), pig (pPRL), horse (ePRL), elephant (elPRL), human (hPRL), rat (rPRL), mouse (mPRL), whale (wPRL), chicken (cPRL) and turkey (tPRL). Growth hormones: bovine (bGH), sheep (oGH), pig (pGH), goat (cGH), horse (eGH), human (hGH), human variant-V (hGHv), rat (rGH), mouse (mGH) and bullfrog (bfGH). Placental lactogens: bovine (bPL) and human (hPL). Sites of bovine prolactin phosphorylation are in **bold**, and residue numbers describe the bovine prolactin sequence.

was not conserved in prolactins or growth hormones from some fish (comparative results not shown).

Analysis of this portion of bovine prolactin by the method of Kyte and Doolittle [55] predicted that serine-90 was the second member of a modestly hydrophilic seven-member sequence (residues 89–95). Analysis by the method of Garnier et al. [56] predicted that serine-90 was located at the elbow of a turn. These predictive algorithms appeared to be consistent with serine-90 being located on the surface of the protein and available to a kinase. Growth hormone was previously identified as a phosphoprotein [9,25,57]. The analogous conserved serine residue may be the site of growth-hormone phosphorylation. The preliminary crystallographic structure for pig growth hormone [58] revealed that the region surrounding this residue was located in helix 2 of the four-helix bundle motif [59].

Phosphorylation of serine at positions 26 or 34 provided a somewhat different picture. Either serine or threonine residues were conserved at both sites in the prolactins examined. These sites were absent from growth hormones and placental lactogens.

We have developed an isolation of phosphorylated and native bovine prolactins and have characterized the products. The range of ratios of these hormone variants quantitatively isolated from individual pituitaries suggested that their synthesis may be a regulated process. Chromatographic and spectroscopic data suggested that phosphorylation was associated with a change in the conformation of the hormone.

With methods developed to purify both native and phosphorylated bovine prolactins, the specific biological actions of these variants may now be elucidated. Completion of such studies may confirm our hypothesis that bovine prolactin can be post-translationally phosphorylated to produce hormone variants with unique biological actions.

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