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PROTEIN KINASE ACTIVITY FROM LACTATING BOVINE MAMMARY GLAND

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SUMMARY

1. Protein kinase activity (ATP:protein phosphotransferase, EC 2.7.1.37) has been detected in crude extracts from lactating bovine mammary gland.

2. This activity has been partially purified and shown to consist of several components, separable by gel filtration.

3. The properties of the two main fractions of enzyme activity have been compared and shown to be similar, however, they may be distinguished by their sensitivity to adenosine 3′,5′-monophosphate.

4. Analysis of the patterns of phosphorylation of the components of whole casein appears to offer a useful method for distinguishing protein kinases with different substrate specificities.

INTRODUCTION

Protein kinase activities (ATP:protein phosphotransferase, EC 2.7.1.37) have been obtained from a variety of sources including bacteria[1] and the tissues of vertebrates[2] and invertebrates[3]. WALSH *et al.*[4] identified a protein kinase from rabbit muscle as being involved in the regulation of phosphorylase activity in that tissue. This enzyme, like most other protein kinases subsequently described, is stimulated by adenosine 3′,5′-monophosphate (cyclic AMP).

In view of these reports attributing regulatory roles to protein kinases from a range of tissues, it seemed of interest to examine the protein kinase activity of lactating mammary gland tissue since this gland excretes large quantities of the phosphoprotein casein. It is known from the work of TURKINGTON AND TOPPER[5] that the phosphate of casein is incorporated subsequent to the synthesis of the polypeptide chains. This tissue might therefore be expected to contain enzymes capable of transferring phosphate groups to proteins which play a strictly structural rather than regulatory role. In this connection it has been demonstrated by PEPPER AND THOMP-

Abbreviations: cyclic AMP, adenosine 3′,5′-monophosphate; SCM-κ-casein, *S*-carboxymethyl-κ-casein.

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SON[6] that the phosphate groups of α_s-casein are essential for the incorporation of α_s-casein into casein micelles.

In this report it is shown that several protein kinase activities are present in extracts prepared from lactating bovine mammary gland. These activities have been partially purified and characterised and their ability to phosphorylate a variety of protein acceptors studied.

MATERIALS AND METHODS

Materials

$(NH_4)_2SO_4$ was 'enzyme grade' from Mann Research Chemicals; mercaptoethanol was the product of Fluka (Switzerland) and was used without further purification. Protamine, prepared from salmon sperm, and cyclic AMP were obtained from Sigma. Sephadex G-200, particle size 40–120 μm, lot No. 3294 was the product of Pharmacia; DEAE-cellulose was the Whatman DE-11 product. Histone (unfractionated) was obtained from Mann. [γ-^{32}P]ATP was prepared by the method of GLYNN AND CHAPPEL[7]. Tris and phosphate buffers were prepared by making approximately molar solutions, adjusting the pH of these to the specified value at 25°, and then making to volume to give 1 M solutions.

Preparation of protein kinases

Lactating bovine mammary gland tissue was obtained from freshly slaughtered animals, cut into small pieces and immediately placed in powdered dry ice. This material was then stored at −20° till use. Homogenisation of thawed tissue was by means of an Ultra-Turrax hand held homogeniser or, when volumes larger than 100 ml were being processed, by means of a Waring blender. The details of the preparation of enzyme from this source are given in RESULTS.

Rabbit muscle protein kinase was prepared from the skeletal muscle of New Zealand White rabbits following the method of WALSH *et al.*[4] except that only one DEAE-cellulose column chromatography step was used. The final preparation contained 8% of the total protein kinase activity present in the crude extract (determined using casein as phosphate acceptor) and was purified approx. 45-fold.

Protein kinase assays

The activity of mammary gland protein kinase fractions was assayed in an incubation volume of 0.2 ml containing, unless otherwise stated: 10 μmoles Tris–HCl buffer (pH 7.5); 4 μmoles NaF; 0.4 μmole theophylline; 0.06 μmole of ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*-tetraacetic acid; 1.2 mg casein; 1.0 μmole magnesium acetate; 0.08 μmole [γ-^{32}P]ATP, containing $4 \cdot 10^5$–$4 \cdot 10^6$ counts/min; 0.05 μmole cyclic AMP; and enzyme. The assay mixtures were incubated for 10 min at 30°. The reaction was initiated by the addition of ATP. The reaction was stopped by the addition of 1 ml of ice-cold 0.1 M sodium pyrophosphate followed by 0.5 ml of cold 25% trichloroacetic acid. The precipitated protein was collected on Whatman GF/C glass fibre filter discs which were then washed with 5% trichloroacetic acid and dried in an oven at 140°. In situations where enzyme activity was low the non-specific adsorption of radioactivity to precipitated casein could be reduced to practically zero by collecting the trichloroacetic acid precipitate by centrifugation, dissolving it in 0.1 M sodium

pyrophosphate and a few drops of 1 M NaOH and then reprecipitating the protein as before. The glass fibre discs were counted either on planchettes in a Nuclear-Chicago Model 181B gas-flow counter or by liquid-scintillation counting in a Packard Tri-Carb Model 2002 scintillation counter. Assays employing histone and protamine as phosphate acceptors were performed similarly except that trichloroacetic acid was added to a final concentration of 25%.

The activity of the rabbit muscle protein kinase was assayed under the conditions described by WALSH *et al.*[4] and acid-insoluble counts determined as described above.

1 unit of enzyme activity is defined as that amount of enzyme which transfers 1 nmole of phosphate to acid-precipitable protein under the assay conditions.

Protein fractions

Whole casein used as phosphate acceptor in routine protein kinase assays and as starting material for the preparation of purified casein components, was obtained from raw bovine milk by acid precipitation. Electrophoretically pure α_s- and β-caseins were prepared by column chromatography of carboxymethylated whole casein as described by MACKINLAY AND WAKE[8]. Dephosphorylated casein fractions were prepared by treatment with bovine spleen phosphoprotein phosphatase (EC 3.1.3.16) which was purified by means of the procedure reported by HOFMAN[9]. A 50-fold purification was achieved compared to the 60-fold purification reported by HOFMAN. The enzyme preparation was tested for possible contamination with proteolytic enzyme activity by incubation with *S*-carboxymethyl-κ-casein (SCM-κ-casein) followed by analysis of the incubation mixture by starch–urea gel electrophoresis. No indication of para-SCM-κ-casein formation or of other proteolysis of SCM-κ-casein was obtained by this means and it was concluded that proteolytic enzyme activity was absent (see refs. 8 and 10). Treatment of casein fractions with spleen phosphatase was carried out in incubation mixtures containing in a volume of 9 ml: 120 mg casein fraction, 3 mg phosphatase, 250 μmoles acetate buffer (pH 6.0), 40 μmoles ascorbate. The mixture was incubated 70 min at 37°. The dephosphorylated casein was recovered and the phosphatase inactivated by the addition of trichloroacetic acid to a final concentration of 2%, the precipitate collected by centrifugation and dissolved in 6 M guanidine hydrochloride and dialysed against dilute Tris buffer (pH 8.0) and finally against distilled water.

Histone fractions f1, f2(a), f2(b) and f3 were prepared from calf thymus glands by Method 2 of JOHNS[11].

RESULTS

Preparation of crude extract

The crude extract containing protein kinase activity was prepared by homogenising thawed minced mammary gland tissue in 2.5 vol. of 0.004 M EDTA (pH 7.0) containing 0.01 M mercaptoethanol. Debris was removed by centrifugation at 16 000 $\times$ *g* for 30 min. The supernatant was decanted through a pad of glass wool to remove fat. Further clarification of the extract was then achieved by centrifuging for 60 min at 68 000 $\times$ *g*. Following this treatment, 95% of the initial activity remained in the

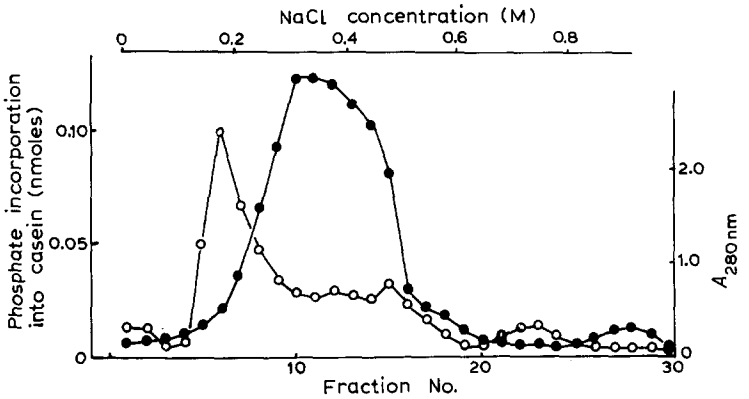

Fig. 1. DEAE-cellulose chromatography of mammary gland protein kinase. 117 $A_{280\,nm}$ units were applied to a 2 cm × 15 cm column in starting buffer containing 5 mM potassium phosphate buffer (pH 7.0), 2 mM EDTA and 10 mM mercaptoethanol. 50% of the protein applied did not adsorb to the column and total recovery was 93%. Elution of adsorbed protein was by means of a linear gradient of NaCl in the concentration range 0–1.0 M. Fraction size: 12 ml. ○—○, $A_{280\,nm}$; ●—●, enzyme activity.

supernatant. The extract was then dialysed against standard buffer (0.004 M EDTA, 0.01 M mercaptoethanol, 0.005 M potassium phosphate buffer (pH 7.0)).

Under conditions of the standard assay, specific activities in the range 0.2–1.0 units/mg protein were obtained for extracts from different samples of tissues. Omission of the protein acceptor from the reaction mixture resulted in levels of incorporation by the crude extract about 30% those obtained with the complete mixture.

Fractionation of the crude extract

Partial purification of the protein kinase activity of the crude extract was achieved by DEAE-cellulose chromatography (see Fig. 1), followed by $(NH_4)_2SO_4$ fractionation. The enzyme fraction precipitating at 35% saturation, called here Fraction I, has been studied without further purification. Another fraction, Fraction II, was obtained by raising the salt concentration to 50% saturation. Fraction II was further resolved by Sephadex G-200 chromatography.

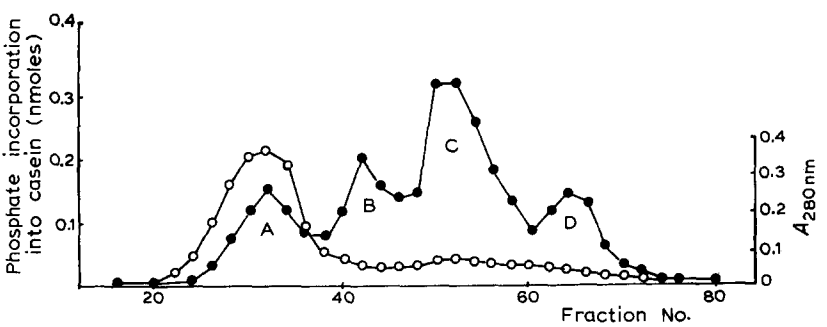

Fig. 2. Sephadex G-200 chromatography of Fraction II. 1.5 ml of the concentrated fraction was applied to a 2 cm × 65 cm column. Fraction size: 1.6 ml; flow rate: 2–3 ml/h. ○—○, $A_{280\,nm}$; ●—●, enzyme activity.

In initial purification attempts salt fractionation was applied directly to the crude extract. It was found however that it was not possible to further purify the salt-fractionated material by DEAE-cellulose chromatography apparently because of aggregation.

Direct chromatography of the crude extract on a 3.5 cm × 24 cm column of Sephadex G-200 resulted in the elution of two peaks of protein kinase activity—a major fraction containing about 80% of the total activity which eluted in the void volume—followed by a second smaller but broader peak. Upon rechromatography both fractions eluted in their original positions. Sephadex G-200 chromatography of each of the two fractions obtained by salt fractionation of the DEAE-cellulose eluate (Fig. 1) showed that these two fractions correspond to the two fractions separable by Sephadex chromatography of the crude extract.

A similar procedure, but employing a Sephadex column with better resolving power was used as the final step in the purification of Fraction II (enzyme precipi-

TABLE I

PARTIAL PURIFICATION OF MAMMARY GLAND PROTEIN KINASES

Enzyme fraction	*Vol. (ml)*	*Protein (mg)*	*Activity (units)*	*Specific activity (units/mg)*	*Purification (-fold)*
Crude extract	640	6802	1690	0.25	1
DEAE-cellulose eluate	110	403	539	1.34	5.4
Fraction I	4.0	144	398	2.80	11.3
Fraction II	4.4	34	72	2.14	8.4
Fraction IIC	6.4	1.0	11.5	11.5	46

tating in 35–50% saturation interval). The elution profile obtained for one such preparation is shown in Fig. 2. Four peaks of enzyme activity, referred to here as Fractions IIA, IIB, IIC and IID, emerge from the column. Fraction IIA elutes in the void volume and this material may resemble Fraction I. The main fraction, IIC, which in this experiment was 45-fold purified, was used for most of the experiments reported here in which its properties have been compared to those of Fraction I. The recoveries of protein and enzyme obtained by the purification procedure outlined above are summarised in Table I.

Characterisation of the enzymically transferred phosphate

The phosphate transferred in the protein kinase reaction was shown to correspond to phosphoprotein phosphate by its lability in 0.25 M NaOH and its stability upon incubation in 0.25 M HCl and 0.1 M NH_2OH.

High-voltage electrophoresis under the conditions used by WILLIAMS AND SANGER[12] was used to demonstrate the presence of ^{32}P-labelled phosphoserine in partial hydrolysates of enzymically phosphorylated casein.

Characterisation of the enyzmic reactions

Time course. The progress of phosphate incorporation into casein was determined using the pooled peak fractions eluted from DEAE-cellulose column and found

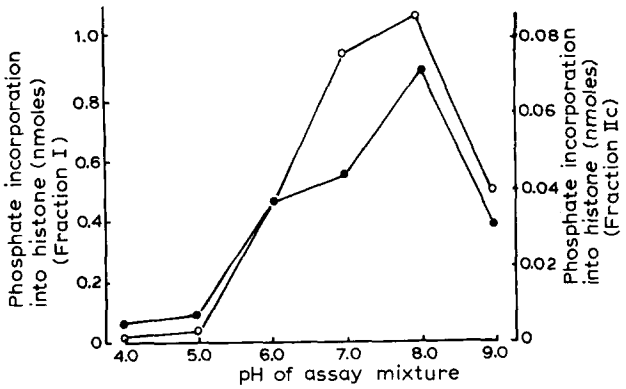

Fig. 3. pH dependence of mammary gland protein kinase fractions. Fraction I (63 μg) and Fraction IIC (4 μg) were assayed in the following 0.05 M buffers: sodium acetate buffer at pH 4.0 and 5.0; sodium glycerophosphate–HCl buffer (pH 6.0); sodium phosphate buffer (pH 7.0); Tris–HCl buffer at pH 8.0 and 9.0. Phosphate acceptor was 0.6 mg unfractionated histone. ○—○, Fraction I; ●—●, Fraction IIC.

to be linear for about 20 min. The rate of incorporation was only slightly lower at the end of 1 h.

pH dependence. The variation of activity with pH for Fractions I and IIC is shown in Fig. 3.

The effect of cyclic AMP. The results of experiments to determine the effect of cyclic AMP on phosphate incorporation into casein, protamine and unfractionated histone by Fractions I and IIC are shown in Fig. 4. It is seen that incorporation by Fraction I is stimulated approx. 4-fold by cyclic AMP where histone is the phosphate acceptor and to smaller extents with casein and protamine. 50% of maximum stimulation was obtained at a cyclic AMP concentration of 0.5 μM. Fraction IIC in contrast is not significantly affected by cyclic AMP. Stimulation by cyclic AMP could not be demonstrated with either of the fractions obtained by direct chromatography of the crude extract on a short column of Sephadex G-200.

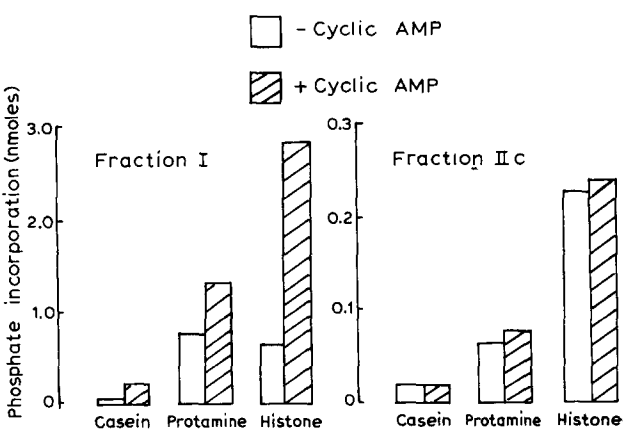

Fig. 4. Effect of cyclic AMP on phosphate incorporation by mammary gland protein kinase fractions. Fractions I (64 μg) and IIC (4 μg) were incubated under standard assay conditions with 1.2 mg of each phosphate acceptor in the presence and absence of cyclic AMP (0.1 mM).

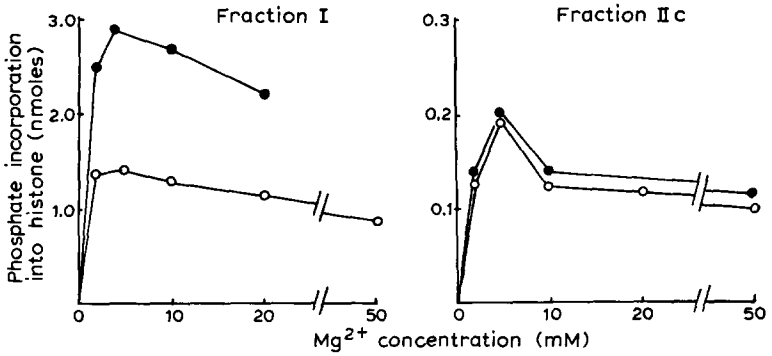

Fig. 5. Dependence of protein kinase activity on Mg^{2+} concentration. Fraction I (63 μg) and Fraction IIC were incubated with f1 histone (0.6 mg) as phosphate acceptor in the presence (0.1 mM) and absence of cyclic AMP. ●—●, + cyclic AMP; ○—○, − cyclic AMP.

The effect of divalent cations. Fig. 5 shows the dependence of Fractions I and IIC on Mg^{2+} concentration when f1 histone was used as acceptor. The effect of cyclic AMP is seen not to vary significantly with Mg^{2+} concentration. Similar results were obtained when casein was used as acceptor.

Ca^{2+} strongly inhibits both kinase fractions, inhibition being complete at 20 mM Ca^{2+}.

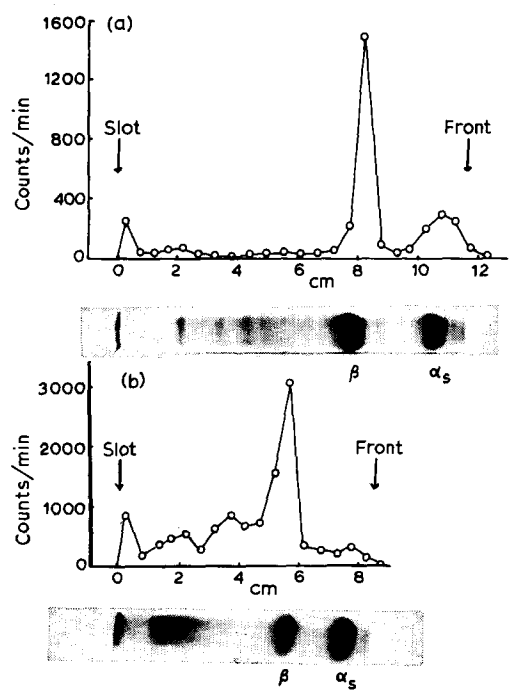

Fig. 6. Radioactivity profiles for whole casein phosphorylated by (a) mammary gland protein kinase (DEAE-cellulose peak fractions) and (b) rabbit muscle protein kinase. Successive gel slices were stained for protein or cut into 0.5-cm pieces for radioactive counting.

The effect of p-hydroxymercuribenzoate. Enzyme Fractions I, IIB and IIC were dialysed to remove mercaptoethanol and then assayed in the presence and absence of 0.4 mM *p*-hydroxymercuribenzoate. Incorporation by these fractions was inhibited by the mercurial to the extent of 87, 59 and 82%, respectively.

Phosphorylation of histone fractions. Enzyme Fractions IIA, IIB and IIC were tested for their ability to phosphorylate calf thymus histone Fractions f1, f2(a), f2(b) and f3. The lysine-rich histones, f1 and f2(b) were excellent phosphate acceptors for all three enzyme fractions, but the arginine-rich fractions f2(a) and f3, were completely resistant to phosphorylation by all three enzyme fractions.

Phosphorylation of casein fractions. In order to characterise the protein kinase activity from mammary gland the pattern of phosphorylation of the major components of whole casein was examined. It seemed of interest to make a comparison in this way between the activity described here and the protein kinase from rabbit muscle described by WALSH *et al.*[4] and thought to be involved in the regulation of phosphorylase activity. Accordingly, samples of whole casein were phosphorylated with the rabbit muscle enzyme, and with the pooled peak fractions of the DEAE-cellulose eluate obtained during purification of the mammary gland enzyme and analysed by starch–urea gel electrophoresis[13]. Fig. 6 shows the staining patterns and radioactivity profiles obtained. The two enzyme fractions give quite distinct patterns of labelling. Both enzymes prefer β-casein as phosphate acceptor, but the mammary gland enzyme phosphorylates α_s-casein to a much greater extent than does the rabbit

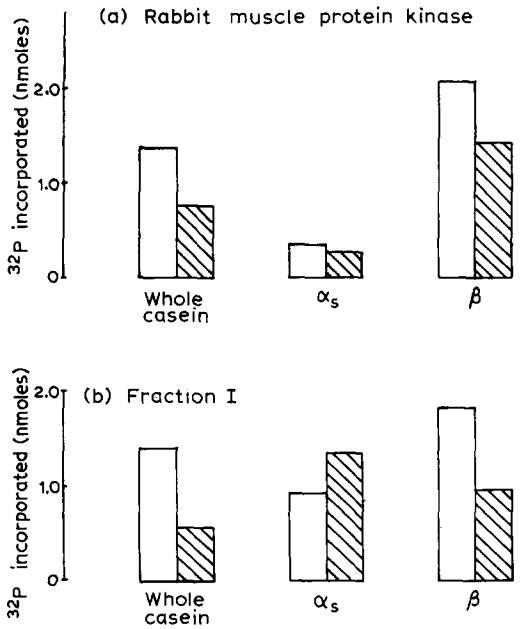

Fig. 7. Phosphorylation of casein by protein kinase preparations. Normal and phosphatase-treated caseins were incubated with enzyme under standard assay conditions. Amount of enzyme per incubation mixture: (a) rabbit muscle protein kinase, 57 μg; mammary gland protein kinase Fraction I, 275 μg. Extent of dephosphorylation after phosphatase teatment: whole casein, 57%; α_s-casein, 77%; β-casein, 83%. Cyclic AMP (0.1 mM) was included in all incubation mixtures. Hatched boxes: phosphatase-treated casein fractions.

muscle enzyme; on the other hand the more slowly migrating κ- and γ-caseins are phosphorylated by the rabbit muscle enzyme but hardly at all by the mammary gland enzyme. In each case the radioactivity peak is located towards the leading edge of the protein band as is to be expected since those protein molecules which have been phosphorylated carry an additional negative charge.

Similar overall results were obtained when mammary gland protein kinase Fraction I and the rabbit muscle enzyme were compared for their ability to phosphorylate whole casein and isolated α_s- and β-caseins. In addition the effect of prior dephosphorylation of these caseins on phosphate incorporation was studied. These results are shown in Fig. 7. It is seen that before phosphatase treatment both enzymes prefer β-casein as phosphate acceptor. However, as in Fig. 6 where whole casein was presented to the enzymes, the mammary gland Fraction I enzyme is able to phosphorylate α_s-casein and this effect is accentuated by prior dephosphorylation of α_s-casein.

DISCUSSION

The mammary gland protein kinases, although purified on the basis of their ability to phosphorylate casein, resemble most of the other protein kinases so far described, including those reported to be present in some 15 other bovine tissues[14], in their preference for basic proteins as phosphate acceptors[2-4]. A recent paper has described the results of a similar study on protein kinases from lactating rat mammary gland[15]. Two activities were separated which were similar in overall properties but distinguishable by their response to cyclic AMP. The rat enzymes resemble those described here in their sensitivity to mercurials, their requirement for Mg^{2+} and inhibition by Ca^{2+}, and in the larger size, as judged by sedimentation, of the cyclic AMP-sensitive enzyme. The rat and bovine enzymes differ in their pH optima, optimum Mg^{2+} concentrations and in their relative preferences for histone and protamine as phosphate acceptors.

The enzyme fractions obtained in this work are but partially purified. In particular Fraction I is only 8-fold purified and may well be resolvable into more than one activity. A better purification was achieved for Fraction IIC, however, polyacrylamide-gel electrophoresis revealed the presence of several protein components in this fraction.

Analysis of the pattern of phosphorylation of the components of whole casein would appear to offer a useful method of distinguishing protein kinases on the basis of their substrate specificity. As the sequences of the major casein components become available (see refs. 16 and 17) it should be possible to localise the amino acid residues phosphorylated and hence define more exactly the specificity of such enzymes. The finding that Fraction I apparently contains an activity which phosphorylates dephosphorylated α_s-casein more readily than α_s-casein may reflect involvement of this activity in casein phosphorylation *in vivo* and this is being investigated further.

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