Purification and Properties of a Ribosomal Casein Kinase from Rabbit Reticulocytes

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A casein kinase was isolated and purified from rabbit reticulocytes. About 90% of the enzyme activity co-sedimented with the ribosomal fraction, whereas about 10% of the enzyme activity was found in the ribosome-free supernatant. Both casein kinases (the ribosome-bound enzyme as well as the free enzyme) showed identical activity and the same molecular weight. On sodium dodecyl sulphate/polyacrylamide-gel electrophoresis a single band of about 70000 mol.wt. was observed. Sucrose-gradient analysis, however, showed that the enzyme activity sedimented with a $s_{20,w}$ of approx. 7.5S. This observation suggested that the casein kinase is a dimer composed of subunits of identical molecular weight. The enzyme utilizes GTP as well as ATP as a phosphoryl donor. It preferentially phosphorylates acidic proteins, in particular the model substrates casein and phosvitin. Casein kinase is cyclic AMP-independent. The K_m values for ATP and GTP with phosvitin as a substrate were determined as 1.2 and 8.8 μM respectively.

Protein kinases that preferentially phosphorylate acidic proteins like casein and phosvitin have been described by numerous authors. These casein or phosvitin kinases have been found in the soluble protein as well as in the microsomal fraction of the cerebral cortex of guinea pigs (Rodnight & Lavin, 1964), in the chromatin fraction of liver (Takeda *et al.*, 1971), in the Golgi apparatus and microsomal fraction of lactating mammary glands (Bingham *et al.*, 1972), in calf brain (Wälinder, 1972, 1973), in the liver of the rooster (Goldstein & Hasty, 1973) and in the ribosomes from rabbit reticulocytes and from wheat embryos (Traugh & Traut, 1974; Carratù *et al.*, 1974). These kinases are present in human lymphocytes (Kemp *et al.*, 1975) and have been found to be part of different animal viruses (Tan, 1975). Desjardins *et al.* (1972) showed the existence of different phosvitin kinases in the rat liver nuclei and the cytoplasmic supernatant. This wide distribution makes it difficult to determine whether these casein or phosvitin kinases have a unique function in the general metabolism or whether they have multiple functions depending on their location in the corresponding cell compartment. However, all casein or phosvitin kinases so far described have at least three distinct features in common: (1) they preferentially phosphorylate acidic proteins; (2) they are not regulated by cyclic AMP; (3) they use GTP as well as ATP as a phosphoryl donor. Despite the

large number of reports on casein kinase activity, only in one case has a phosvitin kinase exhibiting the above described features been purified to homogeneity (Goldstein & Hasty, 1973).

Recently it was shown that cyclic AMP-independent protein kinases appear to be involved in the process of initiation of protein synthesis. Kramer *et al.* (1976) and Ranu *et al.* (1976), who are investigating the mechanism of initiation during protein synthesis, found protein kinases with different specificities to be associated with one initiation factor fraction. Issinger *et al.* (1976) showed that the ribosome-associated casein kinase from rabbit reticulocytes phosphorylated two eukaryotic initiation factors *in vitro*. From this point of view it seemed feasible to purify the ribosome-associated casein kinase activity from rabbit reticulocytes in order to find more intrinsic information about this enzyme which seems so closely connected to the ribosome.

Materials and Methods

Preparation of reticulocytes and ribosomes

Polyribosomes. The procedure of obtaining anaemic rabbits by subcutaneous injection of phenylhydrazine was the same as that described by Allen & Schweet (1962) and Collier (1967). The preparation of the polyribosomes and the high-speed supernatant was carried out as described by Kazemie *et al.* (1973).

40S and 60S subunits. The concentration of the polyribosomes was adjusted to at least 150 A_{260}

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units/ml. Portions (1 ml) were diluted with dissociation buffer, yielding a final concentration of 10 mM-Tris/HCl, pH 7.4, 500 mM-KCl, 5 mM-MgCl₂, 5 mM-dithioerythritol and 0.5 mM-puromycin (Blobel & Sabatini, 1971). Incubation was at 37°C for 30 min. The samples were chilled and loaded on to a hyperbolic sucrose gradient as described by Howard *et al.* (1975), except that a Ti XV zonal-type rotor (Beckman) was used for the separation of the subunits.

Polyacrylamide-gel electrophoresis

The SDS (sodium dodecyl sulphate)/polyacrylamide-gel electrophoresis was carried out as described by Maizel (1969) and as described by Issinger & Traut (1974) and Issinger *et al.* (1975). Disc-gel electrophoresis was as described by Davis (1964) with 5% (w/v) polyacrylamide gels at pH 8.6. When electrophoresis was carried out under non-denaturing conditions (no SDS), gels were run in the cold (4°C) at 2 mA/tube.

Density-gradient centrifugation

The sedimentation coefficient of casein kinase was determined by the procedure of Martin & Ames (1961), in which a comparison of its rate of sedimentation in sucrose gradients was made with that of proteins of known *S* values. Linear gradients of 5–20% (w/v) sucrose in buffer I [20 mM-Tris/HCl (pH 7.2)/100 mM-NaCl/0.1 mM-EDTA/10 mM-2-mercaptoethanol] were loaded with samples containing casein kinase, standard markers or both. Centrifugation was carried out in a Beckman SW41 rotor with Spinco model L2-65B.

Protein determination

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin (fraction V; Sigma Chemical Co., St. Louis, MO, U.S.A.) as a standard.

Preparation of [³²P]ATP and [³²P]GTP

The method of Glynn & Chappell (1964) was used. [³²P]GTP was prepared as described by Issinger & Traut (1974). The specific radioactivity of the [³²P]ATP was about 60 000 c.p.m./pmol and that of the [³²P]GTP about 20 000 c.p.m./pmol.

Protein kinase assays

Cyclic AMP-dependent protein kinase. The reaction volume was 80 µl containing (final concns.): 10 mM-MgCl₂; 50 mM-Mes buffer (4-morpholine-ethanesulphonic acid), pH 6.9; 10 mM-dithioerythritol; 2 nmol of [³²P]ATP (diluted to a specific radioactivity of 3000 c.p.m./pmol); 10 µM-cyclic AMP. When histone IIa (Sigma) was used as a model substrate, 60 µg was used per assay. The reaction mixture contained variable amounts of enzyme

solution. The reaction was carried out at 37°C for 15 min unless otherwise specified.

Casein kinase. The reaction mixture was the same as for the cyclic AMP-dependent protein kinase, except that cyclic AMP was omitted and 130 mM-KCl included. A sample (60 µg) of casein or phosvitin (both reagents from Sigma) was present in each assay. The reaction was stopped by pipetting the whole reaction mixture on to filter squares as described by Corbin & Reiman (1974) and processed accordingly. Radioactivity was counted in toluene/2,5-diphenyloxazole/1,4-bis-(5-phenyloxazol-2-yl)-benzene scintillation fluid. The enzyme activity was expressed in units (1 unit corresponds to 1 nmol/min per mg of protein at 37°C).

Purification of the enzyme from the ribosome-free supernatant

Step 1: (NH₄)₂SO₄ precipitation of crude extract. The ribosome-free high-speed supernatant was brought to 60% saturation by the addition of solid (NH₄)₂SO₄. The pH was maintained between 7 and 8. Protein was allowed to precipitate for 60 min in the cold. It was centrifuged in a Sorvall SS34 rotor for 30 min at 16 000g. The pellet was dissolved in buffer I. This solution was then dialysed overnight against buffer I. The protein concentration was 160 mg/ml. A portion (20 ml) of this material was mixed with glycerol to give 10% (v/v) final concentration.

Step 2: gel filtration. This material was loaded on to a column (2.5 cm × 120 cm) of Sephadex G-200 that had been calibrated with buffer I. Fractions (5 ml) were collected. The casein kinase activity was eluted after 18 h. Fractions 43–49 were pooled (85 ml) and dialysed against buffer I.

Step 3: batch ion-exchange chromatography (phosphocellulose) (Whatman P11) in the same buffer. The casein kinase activity was recovered by a 200 ml gradient of 500–1500 mM-NaCl. Fractions (5 ml) were collected. After the gradient elution the column (2 cm × 30 cm) was washed with 75 ml of 500 mM-NaCl in buffer I followed by a washing step, 1.5 M-NaCl in buffer I. However, no more active material was eluted. Fractions 35–40 were pooled and dialysed against buffer I.

Step 4: batch ion-exchange chromatography (DEAE-cellulose). About 30 ml of pooled material from the phosphocellulose column was batch-adsorbed on DEAE-cellulose (Whatman DE52). The material was then poured into a column (1.5 cm × 30 cm) which was washed with 30 ml of buffer I. There was always some activity in the column wash fractions. The enzyme was eluted with a linear gradient of 150 ml of 0.1–0.3 M-NaCl in buffer I. All of the activity was released by the gradient. Fractions (15 ml) were collected. Further elution with more concentrated NaCl did not result in elution of additional enzyme activity. The peak of activity was

eluted at fractions 22–24. The pooled material from the DEAE-cellulose column was dialysed against buffer II [20 mM-Tris/HCl (pH 7.2)/10 mM-NaCl/0.1 mM-EDTA/14 mM-2-mercaptoethanol] and was then concentrated 15-fold by ultrafiltration on Amicon Difo PM10 filters. The material was dialysed against buffer I.

Step 5: sucrose-gradient analysis. This fraction was applied to a linear sucrose gradient of 5–20% (w/v) sucrose in buffer I and was centrifuged for 22 h at 95000g at 4°C in a Beckman SW41 rotor. The gradient was fractionated in 0.5 ml portions and 50 µl samples were assayed for casein kinase activity. The peak fractions were pooled and dialysed against buffer I.

Purification of the casein kinase from 80S ribosomes

Step 1: removal of enzyme activity from the ribosomes. Between 130 mg and 1 g of polyribosomes were isolated as described and dialysed against 50 mM-Tris/HCl (pH 7.5)/500 mM-KCl/5 mM-MgCl₂/7 mM-2-mercaptoethanol at 4°C. After the dialysis the material was incubated at 37°C in the presence of 0.5 mM-puromycin for 20 min, followed by centrifugation in a Beckman Ti 60 rotor at 140000g at 4°C for 5 h. The ribosome-free supernatant was removed and dialysed against buffer I.

Step 2: (NH₄)₂SO₄ precipitation and sucrose-gradient analysis. The material from Step 1 was concentrated by the addition of solid (NH₄)₂SO₄. The conditions were as described above. Dialysis was against buffer III [50 mM-Tris/HCl (pH 7.5)/80 mM-KCl/5 mM-MgCl₂/7 mM-2-mercaptoethanol] followed by sucrose-gradient analysis as described above. The main peak fractions of activity were pooled and dialysed against buffer I.

Step 3: phosphocellulose chromatography. The material from Step 2 was applied to a phosphocellulose column (2 cm × 30 cm) that had been equilibrated with buffer I. After application of the enzyme, the column was eluted as described above. The main peak fractions of casein kinase activity were collected, pooled and retained. A sample (3.5 ml) of the material was concentrated 7-fold by ultrafiltration on Amicon PM10 filters and then dialysed

against buffer IV [5 mM-Tris/HCl (pH 7.5)/25 mM-NaCl/2 mM-2-mercaptoethanol]. A portion (200 µl) of this sample was freeze-dried and resuspended in 50 µl of double-distilled water.

Step 4: polyacrylamide-gel electrophoresis. The sample was then analysed by disc-gel electrophoresis (no SDS) (Davis, 1964). After electrophoresis the gel was sliced in the cold into 12 pieces. Each single piece was transferred into a small tube and 200 µl of crushing buffer was added [10 mM-Mes buffer (pH 6.9)/3 mM-dithioerythritol] and the mixture was incubated for 1 h at 4°C. The gel pieces were crushed with a glass rod and incubated for another hour. Then the gel tubes were incubated for 30 min at 37°C. After the treatment the material was poured on to a Buchner funnel, vacuum was applied and the liquid material was removed from the crushed gel pieces. A sample (20 µl) of the liquid was used as the enzyme source. Fig. 6(a) shows a typical distribution of the enzyme activity over the analysed polyacrylamide-gel slices.

Fig. 1. *Sephadex G-200 chromatography of fraction (I)* Fraction (I) obtained by the (NH₄)₂SO₄ fractionation was chromatographed on a column (2.5 cm × 120 cm) of Sephadex G-200 as described in the Materials and Methods section. Fractions (5 ml) were collected and 20 µl samples assayed for casein kinase activity. Protein concentration was monitored by determining the A₂₈₀ (○). The casein kinase activity was expressed in pmol of ³²P incorporated/min per µl of fraction (●).

Table 1. *Purification of casein kinase from ribosome-free supernatant of rabbit reticulocytes*

Casein kinase fractions from the different purification steps were assayed as described in the Materials and Methods section. A unit of activity is defined as the amount (nmol) of ³²P incorporated per min.

Fraction	Total protein (mg)	Specific activity (units/mg)	Total activity (units)	Purification (fold)	Yield (%)
(I) (NH ₄) ₂ SO ₄	3000	0.087	261	1	100
(II) Sephadex G-200	460	0.539	248	6	95
(III) Phosphocellulose	41	4.814	197	55	75
(IV) DEAE-cellulose	2	32.000	64	370	24

Step 5: SDS/polyacrylamide-gel electrophoresis. Gel-slice six which contained the highest casein kinase activity was smashed with a glass rod in the presence of SDS sample buffer and incubated for 2h in a 37°C-water bath. The material was heated for 90s in a boiling-water bath and was then subjected to analysis by SDS/polyacrylamide-gel electrophoresis in a 10% gel (Fig. 6b).

Results

Enzyme purification

The purification procedure gave reliable purification, yielding from both sources (ribosomes and ribosome-free supernatant) material of the same

specificity. Table 1 shows a summary of casein kinase purification from the ribosome-free supernatant. The elution profiles of casein kinase activity on Sephadex G-200, phosphocellulose and DEAE-cellulose are shown in Figs. 1, 2 and 3 respectively. The sucrose-density-gradient analysis of the purified enzyme revealed a $s_{20,w}$ value of 7.5 S. SDS/polyacrylamide-gel analysis of the different purification steps are shown in Fig. 4. Fig. 4 (E) shows the protein pattern obtained after chromatography of fraction (IV) on Sephadex G-100. As this purification step led to great losses of material, it was omitted during all further purifications. Fraction (IV) was always directly analysed on sucrose gradients. The material that was derived from the sucrose-gradient analysis,

Fig. 2. Phosphocellulose chromatography of fraction (II)

Fraction (II) was batch-adsorbed on phosphocellulose and poured into a column (2cm × 30cm). Casein kinase activity was eluted with a linear NaCl gradient (—) as described in the Materials and Methods section. Fractions (5ml) were collected and samples (20µl) were assayed for casein kinase activity (●). Protein concentration (○) was determined by measuring the A_{280} . The casein kinase activity was expressed in pmol of ^{32}P /min per µl of enzyme fraction.

Fig. 3. DEAE-cellulose chromatography of fraction (III)

Fraction (III) was batch-adsorbed on DEAE-cellulose. The material was then poured into a column (1cm × 30cm) and eluted by a linear NaCl gradient (—) as described in the Materials and Methods section. Samples (50µl) of the 1.5ml fractions were assayed for casein kinase activity (●). The protein concentration of each column fraction was determined by measuring the A_{280} (○).

Fig. 4. SDS/polyacrylamide-gel electrophoresis of casein kinase from the ribosome-free supernatant at six different stages of purification

Electrophoresis was from the cathode (top) to the anode (bottom). The preparations of casein kinase shown are: A (fraction I); B (fraction II); C (fraction III); D (fraction IV); E (pooled material of the Sephadex G-100 step which followed DEAE-cellulose chromatography); F (protein pattern as it is obtained after sucrose-gradient analysis of the DEAE-cellulose-derived material fraction IV). For details of the fraction see the text.

Fig. 5. Sucrose-gradient centrifugation of unwashed 80S ribosomes

A sample (8 A_{260} units) of 80S ribosomes were layered over a 5–16% sucrose gradient in buffer (V) and centrifuged at 174000g for 230min at 4°C in a Beckman SW41 rotor. The gradient was fractionated into 0.5ml fractions. Samples (10μl) of the sucrose-gradient fractions were assayed for casein kinase activity. The casein kinase activity (○) was expressed in pmol of ^{32}P incorporated/min per μl of enzyme fraction. The A_{260} profile (●) shows the distribution of the ribosomes.

the last step involved in the purification scheme, exhibited only one band of about 70000mol.wt. on SDS/polyacrylamide-gel electrophoresis (Fig. 4, F). To demonstrate the association of casein kinase with the ribosomes, polyribosomes were centrifuged on a linear sucrose gradient. The gradient was fractionated and analysed for absorption and casein kinase activity. Fig. 5 shows that the enzyme activity is identical with the ribosomal peak measured at A_{260} . After removal of the enzyme activity from polyribosomes [0.5M-KCl-washing step at 37°C as described by Blobel & Sabatini (1971)] a sucrose-gradient analysis of the removed material was carried out. The S value of the main peak of activity of the crude salt wash was about 7.5S and thus

identical with that of the purified enzyme from the ribosome-free supernatant. However, there were also minor peaks of activity with higher sedimentation values, indicating the presence of multimeric forms of casein kinase. These multimeric forms were only observed in the crude unfractionated KCl wash from polyribosomes (results not shown). Fractions from the main peak of activity were pooled and the material was applied to a column (2cm×30cm) of phosphocellulose. Column chromatography was carried out under the same conditions as those described in the legend to Fig. 2. The most active fraction from the phosphocellulose column was concentrated by Amicon ultrafiltration and analysed by disc-gel electrophoresis (non-denaturing conditions; Davis, 1964). Fig. 6(a) shows the distribution of the enzyme activity over the sliced disc gel. The main enzyme activity was extracted from the gel and

Fig. 6. Disc-gel electrophoresis of ribosome-bound casein kinase after phosphocellulose chromatography. (a) Electrophoresis of the casein kinase was carried out as described in the Materials and Methods section and the gel was sliced into 12 pieces. Each piece was analysed for casein kinase activity. (b) Gel-slice six from the disc gel in (a) was extracted and the material applied to an SDS/polyacrylamide gel and analysed as described in the Materials and Methods section.

analysed by SDS/polyacrylamide-gel electrophoresis (Fig. 6b). A protein band with a mol.wt. of about 70000 (Fig. 6b) showed up after gel electrophoresis on an SDS/polyacrylamide gel. A comparison between the amount of free and ribosome-bound casein kinase activity in reticulocytes was carried out. Polyribosomes (1.0g) yielded a total enzyme activity of $1.4 \mu\text{mol/min}$. Protein (1.0g) of the ribosome-free supernatant yielded approx. $0.1 \mu\text{mol/min}$ of enzyme activity. Thus about 90% of the casein kinase activity seems to be associated with the polyribosomes whereas only 10% was found in the supernatant. This is a rough calculation and may vary considerably. Extended studies are necessary to determine the exact distribution ratio between free and bound casein kinase activity.

Characterization of the enzyme

Time-dependent phosphorylation. Incubation of either phosvitin or casein with the enzyme led to a constant incorporation of ^{32}P which was linear up to 120min incubation time. Phosvitin seemed to be a better substrate than casein (Fig. 7). However, no implication should be made here whether casein or phosvitin is the better substrate for the casein kinase.

The phosphorylation is dependent on the amount of internal phosphate already present in each of the commercial substrates. All enzyme assays were carried out in duplicate. The values obtained differed by $\pm 10\text{--}20\%$; average values are shown.

Phosphorylation of different substrates. To check

Fig. 7. Time-course of phosphorylation. Incubation was carried out as described in the Materials and Methods section. Δ , Phosvitin ($60 \mu\text{g}$); $\bullet$, casein ($60 \mu\text{g}$); $\circ$, no substrate.

Table 2. Substrate specificity of casein kinase

The casein kinase activity was determined as described in the Materials and Methods section. A sample ($50 \mu\text{g}$) of each protein was tested as substrate. All assays were carried out in the presence ($10 \mu\text{M}$) and in the absence of cyclic AMP. Background for all proteins listed, except phosphorylase B kinase, was approx. 2 pmol and has been subtracted. Background for phosphorylase B kinase was about 40 pmol . All enzyme tests have been carried out in duplicate. The values obtained usually differed by no more than $10\text{--}20\%$. Phosphorylase B and phosphorylase B kinase were a gift from Dr. E. G. Krebs. All other proteins listed below were from Sigma.

	Casein kinase activity (pmol of ^{32}P incorporated/min per μl of enzyme)	
	+ Cyclic AMP	– Cyclic AMP
Phosphorylase B kinase	0	0
Phosphorylase B	0	0
Insulin	0	0
Histone IIa	0.16	0.14
Casein	8.50	9.50
Phosvitin	16.50	15.00
Bovine serum albumin (fraction V)	0	0
80S ribosomes from rabbit reticulocytes	3.00	3.90

the purity of the enzyme, that is to rule out the presence of catalytic amounts of cyclic AMP-dependent protein kinase, I tried to phosphorylate phosphorylase B kinase (a gift from Dr. E. G. Krebs, University of California, Davis). All assays were carried out in the presence and in the absence of cyclic AMP. Table 2 shows that no phosphorylation of phosphorylase B kinase occurred. It should be mentioned that phosphorylase B kinase incorporated about 40 pmol of ^{32}P in the absence of any enzyme. This value was therefore subtracted. All other substrates listed in Table 2 incorporated about 2 pmol of ^{32}P . This value was also subtracted as a common background.

Effect of substrates and nucleotide triphosphate concentration on phosphorylation. When phosphorylation of phosvitin was catalysed by the casein kinase an apparent Michaelis constant, K_m , can be calculated for this substrate. It was determined to be 0.16 mg/ml (4.7 μM). The K_m values for ATP and GTP with phosvitin as a substrate (concentration of phosvitin remains constant) were determined to be 1.2 and 8.8 μM respectively. The kinetic constants were obtained from double-reciprocal plots.

Discussion

The present results show that about 90% of the casein kinase activity found in rabbit reticulocytes is associated with the polyribosomes. The bound protein kinase activity was lost on dissociation of the ribosomes in high salt (0.5M-KCl). The ribosome-associated casein kinase in rabbit reticulocytes shows three distinct features: (1) it preferentially phosphorylates acidic proteins; (2) it is not regulated by cyclic AMP; (3) it uses GTP as well as ATP as a phosphoryl donor. These features distinguish this enzyme from the cyclic nucleotide-dependent protein kinases that play such a decisive role in certain metabolic pathways [e.g. glycogenolysis; for review see Krebs (1972)]. Rodnight & Lavin (1964), who were among the first to describe the occurrence of a phosvitin kinase in brain tissue, found that most of the enzyme activity was located in the soluble protein fraction. However, they also found considerable amounts of enzyme activity in the microsomal fraction. Goldstein & Hasty (1973) isolated a phosvitin kinase from the liver of the rooster. This enzyme had a K_m value of 7.7 μM for ATP with phosvitin as a substrate. Under similar conditions the reticulocyte enzyme showed a K_m value for ATP of 1.2 μM . Takeda *et al.* (1971) and Desjardins *et al.* (1972) who studied the protein kinases present in rat liver nuclei and rat liver cytosol respectively, also found casein kinase activities showing characteristics that agree well with those found for the enzyme described here. Desjardins *et al.* (1972) could show by means of sucrose-density-gradient analysis that

the cytoplasmic casein kinase activity sedimented at a value of about 7.5S accompanied by a minor peak at 5S. The same values were found here for the highly purified reticulocyte enzyme. However, analysis of the crude ribosomal wash by sucrose-gradient analysis showed that the enzyme activity was distributed all over the gradient. The mol.wts. of the different peaks were 500000, 240000, 140000 and 55000 (results not shown). This last finding agrees with the studies of Baggio & Moret (1972) and Wälinder (1973), who demonstrated the occurrence of multimeric forms of phosvitin kinases in rat liver and calf brain. These multimeric forms of the enzyme also have been found in crude extracts from HeLa cells and mouse ascites-tumour cells (O.-G. Issinger, unpublished results).

The second type of ribosome-associated protein kinases is cyclic AMP-dependent and strictly phosphorylates only histone as a model substrate. ATP is the only usable phosphoryl donor. It is very likely that this kind of protein kinase is related to, if not identical with, the well-studied R_2C_2 protein kinase involved in glycogenolysis. Into the same category one has to count the catalytic subunit 'C', which is not cyclic AMP-regulated but still shows all the features of the holoenzyme. It can only use ATP as a phosphoryl donor and preferentially phosphorylates histones. It would be feasible to test any further newly found protein kinase for its ability to use GTP as a phosphoryl donor when it is not cyclic AMP-dependent in order to achieve a clear-cut classification and in order to avoid confusion as to whether it is a casein kinase or simply the 'C'-subunit of a cyclic AMP-regulated enzyme. Ranu & London (1976) and Kramer *et al.* (1976) showed that the haem-regulated translational inhibitor (HRI), which phosphorylates the small polypeptide (38000 mol.wt.) of the eIF2 system, is a cyclic AMP-independent protein kinase that does not phosphorylate histones. Issinger *et al.* (1976) found that the higher-molecular-weight polypeptide (52000 mol.wt.) of the eIF2 system was phosphorylated by the casein kinase and the cyclic AMP-dependent protein kinase. Thus it seems as if another, third, type of protein kinase is associated with the protein-synthesizing system in eukaryotes.

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