Two protein kinases have been isolated from calf thymus and purified to near homogeneity as judged by polycrylamide gel electrophoresis. The enzymes were resolved by selective adsorption to DEAE-cellulose; the species that did not bind was designated casein kinase I and the bound species was designated casein kinase II. They were further purified by chromatography on CM-cellulose, phosphocellulose, and hydroxylapatite. The final step in the purification of casein kinase I involved chromatography on Affi-Gel Blue. Phosvitin, casein, and non-histone chromatin proteins were actively phosphorylated by both casein kinases I and II. Basic proteins such as histones or protamine were not phosphorylated to an appreciable extent. Casein kinase I utilized ATP as phosphate donor, whereas casein kinase II utilized either ATP or GTP. \( K_m \) values of 22 \( \mu \)M for ATP for casein kinase I and 14 \( \mu \)M ATP and 30 \( \mu \)M GTP for casein kinase II were determined in the presence of 5 mM MgCl₂. The enzymes were not stimulated by cAMP or cGMP.

A sedimentation constant of 3.2 S was determined for purified casein kinase I. Polycrylamide gel electrophoresis in the presence of sodium dodecyl sulfate resulted in a single band of 37,000 daltons. Incubation of enzyme in the presence of magnesium and ATP resulted in the incorporation of 2–4 mol of phosphate/m of casein kinase I. Following phosphorylation, 5 species of casein kinase I were resolved by polycrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

A sedimentation constant of 7.8 S was determined for purified casein kinase II. Polycrylamide gel electrophoresis in the presence of sodium dodecyl sulfate showed the enzyme is composed of subunits of molecular weights 44,000, 40,000, and 26,000. Incubation of casein kinase II in the presence of magnesium and ATP resulted in the phosphorylation of the 26,000-dalton subunit.

Protein phosphorylation has been shown to play a role in the regulation of a variety of cellular processes (see recent reviews by Greengard, 1978; Krebs and Beavo, 1979). Indeed, considerable information is available concerning the structure and function of cAMP-dependent protein kinases. Relatively little is known concerning the structure and function of that class of protein kinases not regulated by cyclic nucleotides. A number of cAMP-independent protein kinases have, however, been partially purified and characterized, including protein kinases from rat liver (Desjardins et al., 1972; Rikans and Ruddon, 1976), rat ascites tumor cells (Dahmus, 1976), human lymphocytes (Kemp et al., 1975), rabbit reticulocytes and erythrocytes (Traug and Traut, 1974; Kumar and Tao, 1975), calf brain (Walisner, 1973), and chick oviduct (Keller et al., 1976). More recently, highly purified cyclic nucleotide-independent protein kinases have been characterized in rat liver (Thornburg and Lindell, 1977; Thornburg et al., 1978), Novikoff ascites tumor cells (Dahmus and Natzele, 1977), rabbit reticulocytes (Hathaway and Traug, 1979), and yeast (Lerch et al., 1975). The physiological function of these enzymes is unknown.

Cyclic nucleotide-independent protein kinases have been implicated in the regulation of protein synthesis both at the translational (Farrell et al., 1977; Levin et al., 1976) and transcriptional (Jungmann and Kranias, 1977) level. Regulation at the transcriptional level may involve the modification of non-histone chromatin proteins (Stein et al., 1974; Klein-smith, 1975) or RNA polymerases directly (Dahmus, 1976; Hirsch and Marletta, 1976; Kranias et al., 1977). An understanding of the role protein phosphorylation plays in the regulation of these processes will require an understanding of the structure, function, and cellular localization of the protein kinases involved.

In this communication, we describe a procedure for the purification of milligram quantities of two cyclic nucleotide-independent protein kinases from calf thymus. These enzymes are characterized with respect to their subunit compositions, nucleotide and protein substrate specificity, and autophosphorylation.

**EXPERIMENTAL PROCEDURES**

**Materials**

Unlabeled ATP and GTP were obtained from P-L Biochemicals. \( [\gamma-^{32}P]ATP \) and GTP were purchased from Amersham. Phosvitin, *Escherichia coli* alkaline phosphatase, beef liver catalase, egg white ovalbumin, protamine sulfate, phosphoserine, and phosphotretrine were purchased from Sigma. Casein obtained from Nutritional Biochemicals was treated as described by Reimann et al. (1971). Bovine serum albumin was purchased from Schwarz/Mann. Calf thymus histone were purchased from Sigma. Non-histone chromatin proteins were prepared as described by Christmann and Dahmus (1981).

Phosphocellulose (P1 and P11), DEAE-cellulose (DE22), and CM-cellulose (CM-32) were purchased from Whatman and preactivated as recommended by the supplier before use. Hydroxylapatite and Affi-Gel Blue were obtained from Bio-Rad.

**Methods**

**Protein Kinase Assay**—The standard incubation mixture of 0.08 ml contained: 25 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 0.5 mM EDTA, 0.125 mM \( [\gamma-^{32}P]ATP \) (specific activity 100 cpm/pmol), 100 \( \mu \)g of phosvitin, and enzyme. Some reactions contained 0.2 mM NH₄Cl and are so designated in the text or figure Legend. Reactions were initiated by the addition of enzyme and incubated for 10 min at 37°C. Reactions were terminated by the addition of 2 ml of cold 3% trichloroacetic acid.
acid and after 15 min were collected on nitrocellulose filters (Millipore HAWP2400). The filters were washed three times with 10-mL portions of 2% trichloroacetic acid and after 15 min were collected on nitrocellulose filters (Millipore HA). autoradiography or sliced into individual tracks and scanned in a Gilford recording spectrophotometer equipped with a linear transport. The molar ratio of subunits was determined from spectrophotometric measurements.

Polyacrylamide Gel Electrophoresis—Sodium dodecyl sulfate polyacrylamide gel electrophoresis was carried out in either 7.5% gels according to the procedure of Weber and Osborn (1969) or 12% gels according to the procedure of Laemmli (1970). Unless otherwise indicated, the Weber and Osborn system was used to estimate molecular weight. Standard proteins included phosphorylase b (94,000), albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), and y-lactalbumin (14,400). Gels were stained overnight in Coomasie blue, destained, and either dried for autoradiography or sliced into individual tracks and scanned in a Gilford recording spectrophotometer equipped with a linear transport.

Velocity Sedimentation—The sedimentation coefficients of casein kinases I and II were determined by sucrose gradient centrifugation as described by Martin and Ames (1961). Linear 5-20% sucrose gradients were prepared in 0.05 M Tris-HCl, pH 7.9, 0.1 mM EDTA, 0.2 mM dithiothreitol, 5 mM MgCl2, and 0.15 M NaCl, and centrifuged at 4°C in the Beckman SW 60 Ti rotor at 485,000 × g for 8 or 18 h for casein kinase II and casein kinase I, respectively. In experiments involving the Weber and Osborn system, fractions were collected through the bottom of the tube and an aliquot of each fraction was precipitated with cold 10% trichloroacetic acid. An additional aliquot was assayed for protein kinase activity as described above. Ovalbumin (3.6 S), bovine serum albumin (4.3 S), E. coli alkaline phosphatase (6.3 S), and beef liver catalase (11.2 S) were used as standards.

Partial Acid Hydrolysis and Analysis for Phosphoserine and Phosphothreonine—32P-Labeled casein kinases I or II were precipitated from sucrose gradients, or directly from the reaction mixture, with 10% trichloroacetic acid in the presence of 0.1 M ammonium chloride. After 30 min the precipitate was removed by centrifugation at 143,000 × g in the Beckman 50 rotor for 1 h.

Removal of RNA Polymerase II

The ammonium sulfate concentration of the supernatant was adjusted to 0.075 M by the addition of buffer A (25) and the supernatant was batch-adsorbed to 400 mL of DEAE-cellulose previously equilibrated with buffer A (25) containing 0.075 M ammonium sulfate. After 1 h the resin was removed by filtration and washed four times with 600-mL aliquots of buffer A (25) containing 0.075 M ammonium sulfate. The first wash was combined with the original effluent. Under these conditions, casein kinases I and II as well as RNA polymerase I do not bind to DEAE-cellulose and are recovered in the effluent. The resin was transferred to a column (5 × 20 cm, diameter × height) and RNA polymerase II eluted with buffer A (25) containing 0.5 M ammonium sulfate. RNA polymerase II can be purified as described by Kedinger and Chambon (1972) or as described by Dahmus (1981).

Concentration of Casein Kinase I, Casein Kinase II, and RNA Polymerase I

The 0.6 M eluant from the phosphocellulose P1 column was diluted to 0.04 M ammonium sulfate and adsorbed to 300 mL of DEAE-cellulose previously equilibrated with the same buffer. The resin was collected by filtration and washed three times with 450-mL aliquots of buffer A (25) containing 0.04 M ammonium sulfate. The first wash was combined with the original effluent which contained casein kinase I. The resin was transferred to a column (5 × 20 cm) and RNA polymerase I eluted with buffer A (25) containing 0.04-0.5 M ammonium sulfate in buffer A (25). Fractions were assayed for both RNA polymerase and protein kinase activity. RNA polymerase I elutes at about 0.07 M ammonium sulfate whereas casein kinase II elutes at 0.135 M. RNA polymerase I can be further purified as described by Wasylyk et al. (1979).

Purification of Casein Kinase I

Phosphocellulose Chromatography—To the combined effluent and first wash from the second DEAE-cellulose column was added 100 mL of phosphocellulose P11 previously equilibrated in buffer A containing 0.04 M ammonium sulfate. After 1 h, the resin was collected by filtration and washed twice with 200-mL aliquots of buffer A containing 0.1 M ammonium chloride. The resin was then transferred to a column (2.5 × 40 cm) and developed with a 3-column volume linear gradient of 0.04-0.5 M ammonium sulfate in buffer A (25). Fractions from the phosphocellulose P11 column were diluted to 0.1 M ammonium chloride by the addition of buffer A and adsorbed onto 200 mL of CM-cellulose. The resin was collected by filtration and washed twice with 100-mL aliquots of buffer A containing 0.1 M ammonium chloride. The resin was then transferred to a column (2.5 × 20 cm) and developed with a 3-column volume linear gradient of 0.1-0.7 M ammonium chloride in buffer A. Casein kinase I elutes at about 0.62 M ammonium chloride.

Carboxymethylcellulose Chromatography—Pooled fractions from the phosphocellulose P11 column were diluted to 0.1 M ammonium chloride and loaded onto a column (50 × 2 cm) and developed with a 6-column volume linear gradient of 0.1-1.2 M ammonium chloride in buffer A.

Hydroxylapatite Chromatography—Fractions containing protein kinase activity were pooled and batch bound to 40 mL of hydroxylapatite. The resin was transferred to a column (15.5 × 23 cm) and eluted with an 8-column volume linear gradient of 0.01-0.7 M potassium phosphate buffer, pH 7.2, containing 0.5 mM dithiothreitol. Casein kinase I elutes at about 0.22 M potassium phosphate.

Affi-Gel Blue Chromatography—The enzyme was applied to a column (9.0 × 8 cm) of Affi-Gel Blue previously equilibrated in buffer A containing 0.3 M NH4Cl. The column was washed with 1 column volume of buffer A containing 0.6 M NH4Cl and developed with a 6-column volume linear gradient of 0.6-3 M NH4Cl in buffer A containing 10 mM ATP. Casein kinase I can be further concentrated by precipitation with 55% saturated (NH4)2SO4.
Sucrose gradient centrifugation can be employed in place of Affi-Gel Blue if nucleotide-free enzyme is required. Buffer composition and centrifugation conditions are as described in a previous section. All Km studies were done with casein kinase I purified by gradient centrifugation. Enzyme can be stored at −80°C or in liquid nitrogen for greater than 3 months with no appreciable loss of activity.

**Purification of Casein Kinase II**

Carboxymethylcellulose and Phosphocellulose Chromatography—The peak of casein kinase II from the DEAE-cellulose column was diluted with buffer A to a final ammonium sulfate concentration of 0.05 M and added to 50 ml of CM-cellulose equilibrated in the same buffer. After 1 h, the resin was collected by filtration and washed with 40 ml of buffer A containing 0.05 M ammonium sulfate. The wash was combined with the effluent and added to 100 ml of phosphocellulose P11. Chromatography on phosphocellulose was carried out exactly as described above for casein kinase I. Casein kinase II elutes at about 0.73 M ammonium chloride.

Hydroxylapatite Chromatography—The purification of casein kinase II by hydroxylapatite chromatography is as described above for casein kinase I. Casein kinase II was concentrated by (NH₄)₂SO₄ precipitation, dialyzed, and stored as described above.

**RESULTS**

**Purification of Casein Kinases I and II**—The early stages of the purification are a modification of the procedure developed for the purification of RNA polymerase I and II (Wasylyk and Chambron, 1979). The latter stages of the purification are modifications of the procedure developed for the purification of protein kinases I and II from Novikoff ascites tumor cells (Dahmus, 1976; Dahmus and Natzel, 1977).

The procedure developed for the purification of calf thymus casein kinases I and II also allows for the purification of RNA polymerase I and II. RNA polymerase II is removed by batch adsorption to DEAE-cellulose from the protamine sulfate supernatant. Phosphocellulose P1 is added to the protamine sulfate supernatant to recover casein kinases I and II, and RNA polymerase I. These three activities are step-eluted from phosphocellulose, the ammonium sulfate concentration reduced by dilution and RNA polymerase I and protein kinase II adsorbed to DEAE-cellulose. Casein kinase I, which under these conditions does not bind to DEAE-cellulose, is recovered by batch adsorption to phosphocellulose P11. Casein kinase II and RNA polymerase I are resolved by gradient elution from DEAE-cellulose. The ammonium sulfate concentration of the pooled casein kinase II peak is reduced by dilution and exposed to CM-cellulose. Under these conditions casein kinase II does not bind but a number of high molecular weight protein contaminants are removed that otherwise persist throughout the purification.

The final step in the purification of casein kinase I is chromatography on Affi-Gel Blue. Protein kinase activity elutes as a single peak coincident with the protein peak (Fig. 1). Polyacrylamide gel electrophoresis of peak fractions in the presence of sodium dodecyl sulfate results in a single polypeptide in these fractions (Fig. 1). The final step in the purification of casein kinase II is chromatography on hydroxylapatite. A single peak of protein kinase activity elutes coincident with the protein peak. The constant specific activity across the peak in both the Affi-Gel Blue column for casein kinase I and hydroxylapatite column for casein kinase II suggests that the major fraction of protein is indeed protein kinase. Table I summarizes the results of a typical purification.

**Molecular Weight and Subunit Composition**—The sedimentation constants of casein kinase I and II were determined by centrifugation of the purified enzyme in linear 5–20% sucrose gradient. Casein kinase I sedimented as a 3.2 S component whereas casein kinase II sedimented as a 7.8 S component. In both cases, the activity peak was coincident with the protein peak. A rough estimate of molecular weight based on these sedimentation constants is 36,000 for casein kinase I and 138,000 for casein kinase II (Martin and Ames, 1961).

Polyacrylamide gel electrophoresis of purified casein kinases I and II in the presence of sodium dodecyl sulfate results in a single protein band for casein kinase I (Fig. 2) and the
The molecular weight of the casein kinase I polypeptide, determined by mobility relative to standards of known molecular weight, is 37,000. The fact that a molecular weight of 36,000 is estimated from the sedimentation constant suggests casein kinase I is composed of a single polypeptide.

The molecular weights of the casein kinase II polypeptides are 44,000 ($\alpha$), 40,000 ($\alpha'$), and 26,000 ($\beta$). The molar ratio of subunits determined from gel scans of Coomassie blue-stained gels is 1:0.70:1.5. The subunit stoichiometry of casein kinase II fractionated by sucrose gradient centrifugation was also examined. The molar ratio of $\alpha:\alpha'$ ranged from as high as 1:2 in the leading edge in one gradient to as low as 1:0.63 in the trailing edge. This suggests the $\alpha$ and $\alpha'$ subunits may be related by limited proteolysis or other chemical modification resulting in altered subunit mobility. Peptide mapping of the $\alpha$ and $\alpha'$ subunits by limited proteolysis as described by Cleveland et al. (1977) suggests the two subunits are structurally related (data not presented). The inclusion of 50 $\mu$g/ml phenylmethylsulfonyl fluoride throughout the purification, however, had little effect on the $\alpha:\alpha'$ ratio. The molar ratio of $\alpha + \alpha':\beta$ is 1:0.84 ± 0.098. If we assume the subunit stoichiometry to be $\alpha_2\beta$, the molecular weight of the holoenzyme would be 132,000–140,000. This is in good agreement with the molecular weight estimated from the sedimentation constant.

**Autophosphorylation of Casein Kinases I and II**—Purified casein kinases I and II catalyze the incorporation of $^{32}$P from $\gamma$-$^{32}$P]ATP into the absence of added protein substrate. Purified casein kinases I and II were incubated separately in the presence of magnesium and $\gamma$-$^{32}$P]ATP and aliquots of the reaction mixtures applied directly to 5–20% sucrose gradients. The gradients were assayed for both protein kinase activity and the position of labeled phosphate (Fig. 4). It is clear that, for both casein kinase I and II, the $^{32}$P incorporated prior to centrifugation sediments with the protein kinase activity. This suggests that the phosphate acceptor is the protein kinase itself rather than a contaminating protein.

Aliquots of $^{32}$P-labeled casein kinases I and II were resolved by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate according to the method of Laemmli (1970). The distribution of $^{32}$P was determined by autoradiography of the dried gel. The apparent molecular weight of casein kinase I using the Laemmli electrophoresis method is 34,000, slightly less than that determined using the Weber and Osborn system. Following phosphorylation, casein kinase I is resolved into multiple bands (Fig. 2b). The major fraction of $^{32}$P is associated with a band of apparent molecular weight 37,700 (Fig. 2c). Incubation under these same conditions, except in the absence of ATP, does not result in an altered mobility.

The kinetics of $^{32}$P incorporation into casein kinase I in the absence of added protein substrate is shown in Fig. 5. Assuming a molecular weight of 37,000 for casein kinase I about 2–4 mcl of phosphate are incorporated/mcl of enzyme. Aliquots were removed at various times, denatured, and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Fig. 6). Following phosphorylation casein kinase I is resolved into five bands with apparent molecular weights of 34,400, 35,500, 36,400, 37,700, and 38,900 (Fig. 6). There is a clear progression of protein and $^{32}$P to bands of decreased mobility as a function of time.

The electrophoretic pattern obtained with casein kinase II is shown in Fig. 3. It is clear that the major phosphate acceptor in casein kinase II is the 26,000 dalton subunit. The mobility of this subunit is not altered by phosphorylation.

Purified casein kinases I and II were phosphorylated in the presence of $\gamma$-$^{32}$P]ATP in the absence of added protein substrate and subjected to partial acid hydrolysis as described by Blyund and Huang (1976). The hydrolysates were analyzed by high voltage paper electrophoresis and the $^{32}$P quantitated as described under “Methods.” Approximately 18% of the
radioactivity incorporated into casein kinase I migrated with phosphothreonine and 4% with phosphoserine. In contrast, 5% of the radioactivity incorporated into casein kinase II migrated with phosphothreonine and 14% with phosphoserine. The remaining \(^{32}\text{P}\) in each case migrated with inorganic phosphate.

**Substrate Specificity of Casein Kinase I and II**—The ability of casein kinases I and II to phosphorylate a variety of protein substrates is shown in Table II. Of the proteins tested, phosvitin was the preferred substrate for both casein kinases I and II. Non-histone chromatin proteins were also actively phosphorylated by both enzymes. Basic proteins such as histones and protamines were not phosphorylated to an appreciable extent.

Experiments were also performed to determine the nucleotide substrate specificity for casein kinase I and II. Initial reaction rates were determined in the presence of various concentrations of either \([\gamma^{32}\text{P}]\text{ATP}\) or \([\gamma^{32}\text{P}]\text{GTP}\) and the
Phosphorylation of various protein substrates by calf thymus casein kinases I and II

The assays were performed under the standard assay conditions in the presence of 0.2 M NH₄Cl and 100 µg of protein substrate except in the case of non-histone chromatin protein when 54 µg was present. Background values determined by incubation of protein substrates in the absence of kinase were less than 2 pmol for each protein substrate except histone which gave a background for 56 pmol. No correction was made for casein kinase I or casein kinase II autophosphorylation. The extent of autophosphorylation was partially dependent on the extent of adsorption of casein kinase I1 to DEAE-cellulose. Each enzyme was made for casein kinase I1 fractionated by the selective adsorption of casein kinase I1 to DEAE-cellulose. 'Each enzyme activity of about 1 µmol of phosphate transferred/min/mg of enzyme. The molecular weight of the recovered phosphate in phosphothreonine appears to consist of a single polypeptide. In the absence of added protein substrate, casein kinase I incorporates 2–4 mol of phosphate/mol of enzyme. Electrophoresis of phosphorylated casein kinase I, according to the procedure of Laemmli (1970), results in the resolution of five protein bands. The fact that there is a time-dependent shift of protein from one band to another and the specific activity of the bands differ suggests the altered mobility is a reflection of different levels of phosphorylation. The significance of this phosphorylation is not clear. It is of interest however that bovine cardiac muscle cAMP-dependent protein kinase incorporates 1 phosphate into each of 2 seryl residues in its cAMP-binding protein (Rangel-Aldao et al., 1979) and that the modified protein has an altered mobility in the Laemmli gel system.

We have previously reported the purification of a cyclic nucleotide-independent protein kinase from Novikoff ascites tumor cells that had similar catalytic and chromatographic properties to calf thymus casein kinase I (Dahmus, 1979). Analysis of the ascites tumor enzyme by sodium dodecyl sulfate gel electrophoresis resulted in the resolution of 85,000- and 27,000 dalton polypeptides. The fact that the 37,000 dalton polypeptide incorporates 32P and has a mobility indistinguishable from that of the calf thymus enzyme suggests that it is the polypeptide responsible for the kinase activity in the ascites tumor preparation (Christmann and Dahmus, 1981). We have found no trace of an 85,000 dalton component in the calf thymus preparations. These enzymes appear homologous to casein kinase I purified from rabbit reticulocytes by Hathaway and Traugh (1979).

Analysis of casein kinase II by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate results in the resolution of peptides of 44,000 40,000, and 26,000 daltons. Casein kinase II appears homologous to the cyclic nucleotide-independent protein kinase purified from Novikoff ascites cells (Dahmus and Natzel, 1977), rat liver (Thornberg and Lindell, 1977) and rabbit reticulocytes (Hathaway and Traugh, 1979). Casein kinase II appears to have a molecular weight of about 138,000 and the subunit structure αββ. The relationship between α and β is not clear although they appear to be closely related polypeptides.

After phosphorylation, casein kinase I was hydrolyzed and the products analyzed by high voltage electrophoresis. About 18% of the 32P recovered in amino acid was in phosphoserine and about 82% in phosphothreonine. Analysis of phosphorylated casein kinase II under the same conditions resulted in about 25% of the recovered phosphate in phosphothreonine and about 75% in phosphoserine. This result may reflect a preference for the phosphorylation of threonine residues by casein kinase I and serine residues by casein kinase II or may simply be a reflection of the amino acid composition of the two kinases.

Casein kinases I and II can also be distinguished on the basis of their substrate specificity. Casein kinase I uses exclusively ATP as phosphate donor whereas casein kinase II can utilize either ATP or GTP. The difference in protein substrate specificity is striking when one examines the phosphorylation of non-histone chromatin proteins or RNA polymerase II (Christmann and Dahmus, 1981; Dahmus, 1981). Hathaway et al. (1979) have reported that apparently homologous enzymes from rabbit reticulocytes phosphorylate specific initiation factors of protein synthesis. These studies also demonstrate a difference in the substrate specificity for the two enzymes.

The ability of casein kinases I and II to phosphorylate non-histone chromatin proteins and RNA polymerase II as well as initiation factors involved in protein synthesis suggests that these protein kinases may participate in the regulation of protein synthesis both at the level of transcription and trans-
laction. If indeed protein synthesis is regulated by a phosphorylation-dephosphorylation mechanism, this process must in turn be regulated.

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