Purification and Properties of a Yeast Protein Kinase†

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ABSTRACT: A protein phosphokinase (EC 2.7.1.1.37) was isolated from baker's yeast (*Saccharomyces cerevisiae*) after a 17,000-fold purification; the purified enzyme is homogeneous according to the criteria of gel electrophoresis and ultracentrifuge analysis. The enzyme has a high isoelectric point of ca. 9 and appears to exist as a monomer with a molecular weight of 42,000 ± 1500. It is neither stimulated by cyclic 3′,5′-AMP, -GMP, -CMP or -UMP nor inhibited by the regulatory subunit of rabbit muscle protein kinase (Reimann, E. M., Walsh, D. A., and Krebs, E. G. (1971), *J. Biol. Chem. 246,* 1986). In the presence of divalent metal ions, preferably Mg^{2+} or Mn^{2+}, the enzyme readily transfers the terminal phosphate group of ATP to phosvitin, α_{S1B}- and β_A-casein and an NH_2-terminal tryptic peptide derived from β_A-casein, but not to protamine, lysine, or arginine-rich histones or to yeast enzymes such as phosphorylase, phosphofructokinase, or pyruvate carboxylase; serine and polyserine were also inactive as phosphate acceptors. K_m values of 0.17 m*M* for β_A-casein and 0.2 m*M* for ATP were determined at 10 m*M* Mg^{2+}. The purified yeast protein kinase also catalyzes the reverse reaction, namely, the transfer of phosphate from fully phosphorylated β_A-casein or its NH_2-terminal peptide to ADP resulting in the formation of ATP. AMP, GDP, UDP, and CDP did not serve as phosphate acceptors in this reaction. As observed by Rabinowitz and Lipmann (Rabinowitz, M., and Lipmann, F. (1960), *J. Biol. Chem. 235,* 1043) both reactions have different pH optima with values of 7.5 for the forward reaction (phosphorylation of the proteins) and ca. 5.2 for the formation of ATP; both are differently affected by salts. Phosphorylation of β_A-casein with [γ-^{32}P]ATP followed by digestion of the labeled protein with trypsin indicated that all the radioactivity was exclusively introduced in an NH_2-terminal peptide possessing the unique sequence: Glu-Ser(P)-Leu-Ser(P)-Ser(P)-Ser(P)-Glu-Glu ... (Ribadeau-Dumas, B., Brignon, G., Grosclaude, F., and Mercier, J.-C. (1971), *Eur. J. Biochem. 20,* 264). By subjecting β_A-casein and its NH_2-terminal peptide to the combined action of almond acid phosphatase and purified yeast protein kinase, it was determined that the phosphorylation and dephosphorylation reactions proceed randomly, i.e., all seryl phosphate residues are equally susceptible and that the rate of phosphorylation decreases drastically as the number of bound phosphate groups in the substrate diminishes.

Protein phosphokinases (EC 2.7.1.1.37) catalyze the transfer of phosphate from ATP to a variety of proteins. While much attention has been drawn recently to the cyclic 3′,5′-AMP-dependent protein kinases which mediate certain hormonal actions (for review, see Krebs, 1972), the cAMP-independent protein kinases which mainly phosphorylate acidic proteins or phosphoproteins such as casein or phosvitin have been less thoroughly investigated. Nonetheless, some of these have been partially purified and characterized, including the protein kinases from brewer's yeast (Rabinowitz and Lipmann, 1960), calf brain (Walinder, 1973), rat liver (Takeda et al., 1971), and the mammary gland (Bingham et al., 1972). This latter enzyme said to be located in the Golgi apparatus phosphorylates casein, while the brain and the liver kinases are probably involved in the regulation of nuclear processes (Langan, 1967) since they phosphorylate nuclear proteins, some of which contain runs of seryl-P residues as found in casein (Ribadeau-Dumas et al., 1971) and phosvitin (Clark, 1973).

This work was initiated as an extension of earlier studies on the evolution of enzymatic control mechanisms. A previous publication reported on the purification and comparative properties of pure yeast glycogen phosphorylase (Fosset et al., 1971), while the accompanying article (Lerch and Fischer, 1975) describes the amino acid sequence of the site involved in the covalent control of the enzyme. In the course of this work, attempts were made to isolate the protein kinase responsible for the conversion of yeast phosphorylase *b* to *a,* starting from crude baker's yeast extracts which were shown to catalyze this reaction as well as the phosphorylation of casein and phosvitin. Since purification of the kinase required innumerable enzymatic assays and pure yeast phosphorylase *b* was scarce, casein was routinely used as substrate. As it turned out, all phosphorylase kinase activity was lost in the course of purification and a different protein kinase, strictly specific toward "acidic" proteins such as casein or phosvitin was eventually isolated. The present article reports on the purification and characterization of this enzyme and focusses in some detail on its substrate specificity and mode of action.

Materials and Methods

Casein (Hammersten quality) and phosvitin were obtained from Nutritional Biochemicals, Cleveland, Ohio. Streptomycin sulfate and phenylmethanesulfonyl fluoride were purchased from Sigma Chemical Corp. Fleischman baker's yeast was a generous gift from Standard Brands, Inc. Sephadex forms were obtained from Pharmacia, Piscataway, N.J., and CM-cellulose (CM-52) was from Whatman; cellulose thin-layer sheets with fluorescence indicator (G-065) were from Eastman-Kodak, N.Y. Carrier-free $^{32}P_i$ was obtained from International Nuclear Corporation, and

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was used to prepare [γ-^{32}P]ATP by the procedure of Glynn and Chappell (1964). Cyclic 3′,5′-AMP, -GMP, -CMP and -UMP and yeast phosphofructokinase were purchased from Boehringer, Mannheim. All other reagents were of the highest grade commercially available.

Enzyme Assay. Since the phosphorylation of casein is reversible, phosphorylase kinase assays were carried out in both the forward and reverse directions. For the forward reaction, transfer of ^{32}P from [γ-^{32}P]ATP into casein at 30° was measured. To 0.5 ml of substrate solution containing 1% casein, 5 m*M* [γ-^{32}P]ATP (specific activity *ca.* 2×10^7 cpm/μmol), 10 m*M* $MgCl_2$, and 1 m*M* EDTA in 0.1 *M* Tris-HCl at pH 7.5 was added 0.5 ml of enzyme diluted in 0.1 *M* Tris-HCl (pH 7.5), containing 0.1% bovine serum albumin. At given times, 0.2-ml aliquots were withdrawn and precipitated with 1 ml of 10% trichloroacetic acid and centrifuged at 2000*g* for 5 min; the pellets were redissolved in 0.2 ml of 0.5 *N* NaOH and reprecipitated by the addition of 1 ml of 10% trichloroacetic acid. The suspension was recentrifuged and the pellets were redissolved in 1 ml of 88% formic acid and counted in a dioxane based scintillant (Bray, 1960) using a Packard TriCarb liquid scintillation spectrometer. One unit of protein kinase activity is defined as the amount of enzyme which catalyzes the transfer of 1 nmol of phosphate from ATP to casein in 1 min at 30°.

For routine assay of column fractions, 0.1 ml of appropriately diluted enzyme was reacted with 0.1 ml of substrate solution for 5 min and precipitated with 1.0 ml of 10% trichloroacetic acid, and the precipitate was passed through glass filter discs (2.4 cm diameter, Schleicher and Schuell, New Hampshire), using a filtration device from Yeda (Rehovot, Israel). The filters were washed with 10 ml of 0.1 *N* sodium pyrophosphate (pH 2.0) followed by 5 ml of 10 m*M* HCl, dried at 108°, and counted in 5 ml of toluene based scintillant (0.25% Omnifluor, New England Nuclear, 20% Methylcellulose in toluene).

For the reverse assay, transfer of ^{32}P to ADP was measured at 30° using a solution of ^{32}P-labeled β_A-casein (10 mg/ml, specific radioactivity of ca. 3×10^6 cpm/μmol), 2 m*M* ADP, 10 m*M* $MgCl_2$ in 0.1 *M* sodium morpholinopropanesulfonate (pH 6.0) as substrate. Aliquots were removed and precipitated with 1 ml of 10% trichloroacetic acid. The tubes were centrifuged at 2000*g* for 5 min and 0.5 ml of the supernatant solutions were counted in the dioxane based scintillant. α_{S1B}- and β-caseins were prepared according to Mercier et al. (1968) starting from whole milk of individual Holstein cows preselected to yield the single genetic β_A and α_{S1B} variants of casein as determined by polyacrylamide disc gel electrophoresis at pH 9.2 in 4.5 *M* urea, according to Kiddy et al. (1963).

Yeast phosphorylase *b* was prepared according to Fosset et al. (1971), rabbit muscle phosphorylase *b* according to Fischer and Krebs (1958), rabbit muscle phosphorylase kinase according to Cohen (1973), and almond acid phosphatase according to Clark (1973). Yeast pyruvate carboxylase was a purified preparation from Dr. M. Utter, Case Western Reserve University, Cleveland, Ohio.

Polyacrylamide gel electrophoresis was carried out in sodium dodecyl sulfate according to Weber and Osborn (1969), and in sodium morpholinopropanesulfonate (pH 6.5) according to Rodbard and Chrambach (1972); 7.5% gels were used throughout. Protein kinase was located by soaking 2.4-mm gel slices overnight in 0.2 ml of 0.1 *M* Tris-HCl–1 m*M* EDTA (pH 7.5), then measuring enzymatic activity on 0.1-ml aliquot solutions.

Amino acid analysis was carried out according to Moore and Stein (1963) using norleucine as an internal standard (Walsh and Brown, 1962). Acid hydrolysis was performed in sealed evacuated tubes after repeated flushings with nitrogen in 5.7 *N* HCl at 108° for periods of 24, 48 and 72 hr; the hydrolyzed samples were analyzed on a Beckman Model 120 C automatic amino acid analyzer.

Protein was measured by the procedure of Lowry et al. (1951) or by absorbance at 280 nm, using a value $A_{280}(1\%)$ of 11.3 based on a refractometric determination of the concentration of the pure enzyme by means of the ultracentrifuge (Babul and Stellwagen, 1969). The absorbancy index was 11.5 when based on the amino acid composition of the enzyme (Hsiu et al., 1964).

Dephosphorylation of β_A-casein and of its NH_2-terminal tryptic phosphopeptide β_A-T1 (Manson and Annan, 1971) was carried out with almond acid phosphatase in 0.1 *M* ammonium formate (pH 5.5) at a 1:50 enzyme/substrate ratio. The reaction was stopped by adding a drop of glacial acetic acid and lyophilization; dephosphorylation was followed by direct phosphate analysis according to Fiske and Subbarow (1925). For total phosphate analysis, aliquots were boiled for 15 min in 1 *N* NaOH and hydrolyzed for 48 hr in 5.7 *N* HCl at 108° in evacuated, sealed tubes; acid-hydrolyzed samples were also subjected to amino acid analysis for determination of protein concentration.

Ultracentrifuge analyses were carried out at 5° in 0.1 *M* Tris-HCl–1 m*M* EDTA (pH 7.5) in a Spinco Model E analytical ultracentrifuge equipped with a titanium AN-H rotor and double sector cells for sedimentation velocity runs; high-speed equilibrium sedimentation experiments were performed according to Yphantis (1964) using 3-mm columns. Plates were measured with a Nikon microcomparator and sedimentation equilibrium data were processed on an IBM 7094 computer using the program of Teller et al. (1969). A partial specific volume of 0.733 ml/g at 20° was estimated from the amino acid composition and corrected to 0.726 ml/g at 5° using the factor of $d\bar{v}/dT + 0.0005$ ml/g per deg (Svedberg and Pederson, 1940).

Results

Purification of Yeast Protein Kinase. All steps were performed at 4° in the presence of 0.1 m*M* phenylmethanesulfonyl fluoride to minimize proteolytic degradation. Unless otherwise stated, the purification procedures were carried out in 0.1 *M* Tris-HCl–1 m*M* EDTA buffer (pH 7.5) referred to as buffer A. Twenty pounds (9.1 kg) of pressed baker's yeast were routinely used in each preparation. Yeast was suspended in 10 l. of distilled water and passed twice through a continuous French press (Manton, Gaulin, Everett, Mass.) at 8000 psi. The suspension was centrifuged at 1200*g* for 30 min and filtered through glass wool to give the "crude extract". Streptomycin sulfate was added to a final concentration of 0.5% and the solution was stirred for 1 hr, then centrifuged at 1200*g* for 30 min. The large pellet was discarded and the pH of the supernatant was adjusted to 7.5 with 2 *M* Tris.

CM-Sephadex Filtration. The solution was passed through two large Buchner funnels containing each approximately 100 g of CM-Sephadex C-50 previously equilibrated in buffer A. The ion exchanger was washed with approximately 20 l. of the same buffer, then kinase was eluted with 1 *M* NaCl in buffer A. The slightly pink eluate was precipitated by adding solid ammonium sulfate to 50% saturation. This suspension was stirred gently overnight and

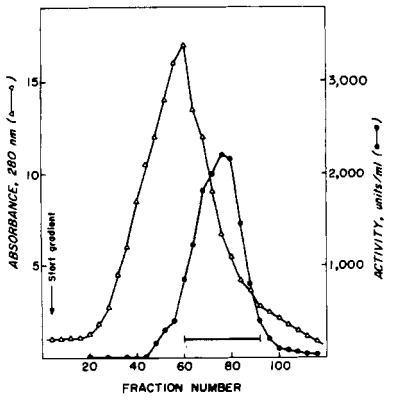

FIGURE 1: CM-Sephadex chromatography of yeast protein kinase. The enzyme was applied to a 4 × 40 cm column of CM-Sephadex C-50 equilibrated with buffer A. The flow rate was 50 ml/hr and 15-ml fractions were collected. A linear gradient of 0–0.5 *M* NaCl in buffer A was run with 800 ml in each chamber. The bar designates the active fraction pooled.

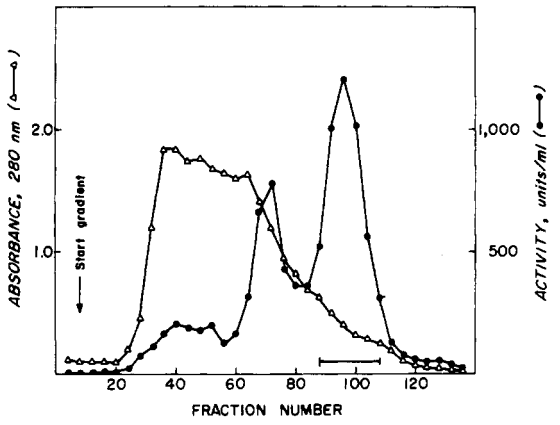

FIGURE 2: CM-cellulose chromatography of yeast protein kinase. The enzyme was applied to a 2.5 × 20 cm column of CM-52 cellulose equilibrated with 0.05 *M* sodium succinate–1 m*M* EDTA (pH 5.8). The flow rate was 40 ml/hr and 8-ml fractions were collected. A linear gradient of 0.05–2 *M* sodium succinate–1 m*M* EDTA (pH 5.8) was run with 500 ml in each chamber. The bar indicates the fractions pooled.

centrifuged at 10,000*g* for 1 hr; the pellet was redissolved in buffer A, dialyzed against several changes of the same buffer, and frozen overnight.

CM-Sephadex Chromatography. The frozen sample was thawed and incubated for 30 min at 30° and the resulting flocculent precipitate removed by centrifugation at 10,000*g* for 15 min; the supernatant was chromatographed on CM-Sephadex C-50 as described in Figure 1. Kinase activity emerged as a single peak at 0.3 *M* NaCl; active fractions were pooled and dialyzed against buffer A.

DEAE-Sephadex and CM-Cellulose Chromatography. To remove neutral contaminants, the solution was applied to a 4 × 20 cm column on DEAE-Sephadex A-50 equilibrated against buffer A; kinase activity appeared in the breakthrough and the active fractions were pooled and immediately dialyzed against 0.05 *M* sodium succinate–1 m*M* EDTA (pH 5.8) (not illustrated).

The dialyzed sample was then applied to a CM-cellulose column and eluted as described in Figure 2. Kinase emerged in two peaks; activity in the first peak varied significantly from one preparation to another but remained constant in the second. Therefore, only the enzyme present in the second peak was collected for further purification.

The eluate was diluted 1:1 with distilled water and concentrated on a 1 × 4 cm column of CM-cellulose. Elution was carried out with 0.5 *M* sodium succinate–1 m*M* EDTA (pH 5.8) and the enzyme was precipitated by adding solid ammonium sulfate to 50% saturation. The precipitate was spun at 20,000*g* for 20 min and the pellet redissolved in buffer A. This solution was dialyzed against several changes of the same buffer; a fine precipitate of inactive material was removed by centrifugation at 20,000*g* for 20 min.

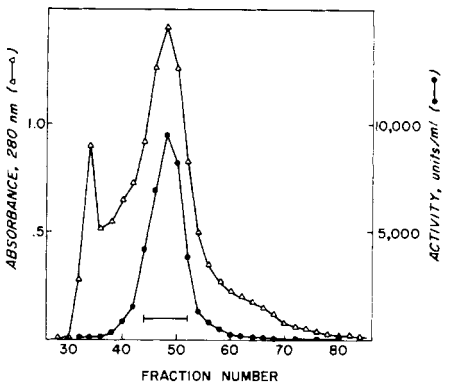

FIGURE 3: G-100 Sephadex gel filtration of yeast protein kinase. A 2-ml sample was applied to a 1.5 × 90 cm column of Sephadex G-100 swollen in buffer A. The flow rate was 15 ml/hr and 1.7-ml fractions were collected. The active fractions indicated by the bar were pooled.

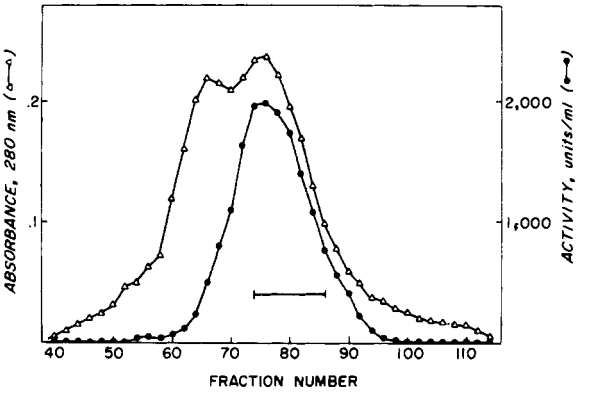

FIGURE 4: Second CM-cellulose chromatography of yeast protein kinase. The enzyme was applied to a 1 × 8 cm column of CM-52 cellulose equilibrated with 0.05 *M* sodium succinate–1 m*M* EDTA buffer (pH 5.8). The flow rate was 20 ml/hr and 1.7-ml fractions were collected. A linear gradient of 0.05–0.2 *M* sodium succinate–1 m*M* EDTA (pH 5.8) was run with 200 ml in each chamber. Fractions with constant specific activity as shown by the bar were pooled.

Sephadex G-100 and CM-Cellulose Chromatographies. The clear supernatant was further chromatographed on Sephadex G-100 as shown in Figure 3; the active fractions were pooled, dialyzed against 0.05 *M* sodium succinate–1 m*M* EDTA (pH 5.8), and chromatographed a second time on CM-cellulose as illustrated in Figure 4; fractions with constant specific activity were pooled, dialyzed against buffer A, and subsequently concentrated by vacuum dialysis. Table I summarizes the results obtained for a typical preparation.

Enzyme Purity. Because the yeast protein kinase is very basic, most buffer systems ordinarily used for gel electrophoresis of proteins could not be applied. The enzyme does not penetrate the gel at pH 8.3; at pH 4.0, it migrates with the dye. Good resolution was achieved in the cathodic sodium morpholinopropanesulfonate system of Rodbard and Chrambach (1972) even though somewhat diffuse bands were generally obtained. The purified yeast protein kinase

Table I: Summary of a Typical Purification of Yeast Protein Kinase.

Step	Volume (ml)	Protein[a] (mg)	Units[b]	Specific Activity	Yield (%)
1. Crude extract	13,000	732,000	410,000	0.56	100
2. Streptomycin sulfate supernatant	11,000	470,000	400,000	0.85	97
3. CM-Sephadex eluate	8,000	28,000	380,000	14.1	92
4. 50% saturated ammonium sulfate + heat step	180	10,500	350,000	33.3	85
5. CM-Sephadex eluate	200	1,220	304,000	250	74
6. DEAE-Sephadex eluate	275	370	240,000	650	58
7. CM-Cellulose eluate	156	70	130,000	1940	32
8. Sephadex G-100	15	15.5	98,000	6400	24
9. Second CM-cellulose chromatography	22	3.8	37,000	9800	9

[a] Protein was measured in steps 1–4 by the procedure of Lowry et al. (1951) and in steps 5–9 by absorbancy using the value A_{280} (1%) of 11.5 as determined on the pure enzyme. [b] Using casein as substrate (see Materials and Methods).

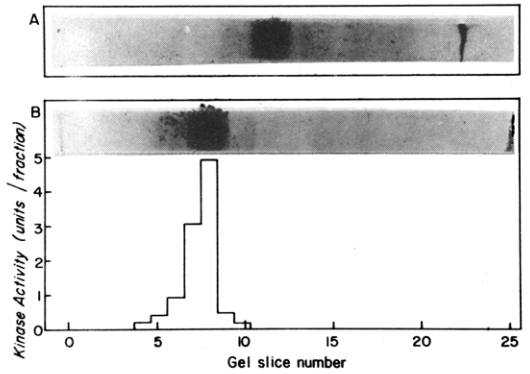

FIGURE 5: (A) Sodium dodecyl sulfate gel electrophoresis of yeast protein kinase on 7.5% polyacrylamide gels; 10 μg was applied and the gel was stained with Coomassie Blue. (B) Polyacrylamide gel electrophoresis in sodium morpholinopropanesulfonate (pH 6.5) on 7.5% gels; 10 μg was applied and the gel was stained with Coomassie Blue. (C) Activity profile on slices of a parallel gel run as described under (B); 40 units (ca. 4 μg) of protein kinase was applied.

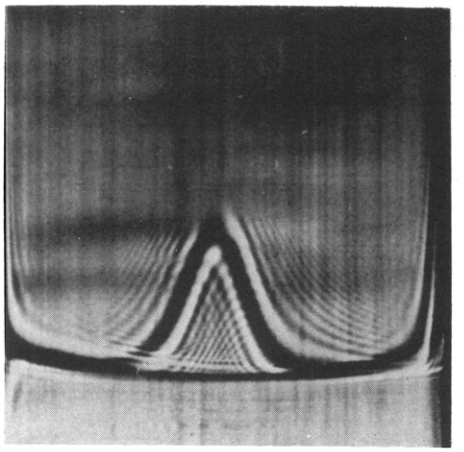

FIGURE 6: Sedimentation velocity ultracentrifugation of yeast protein kinase (3.9 mg/ml) in buffer A at 5°, 67,000 rpm. The picture was taken 32 min after speed had been attained. Sedimentation is from left to right.

gives a single band on polyacrylamide gel electrophoresis in both sodium dodecyl sulfate (Figure 5A) and on sodium morpholinopropanesulfonate (pH 6.5) (Figure 5B); Figure 5C shows the activity profile of such a gel determined as indicated under Materials and Methods. It also gives a single symmetrical peak in the analytical ultracentrifuge (Figure 6) with an $s_{20,w} = 2.9 \pm 0.1$ S.

Molecular Weight. The molecular weight of yeast protein kinase was determined both by high-speed sedimentation equilibrium analysis and polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. In the former case, a weight average molecular weight for three concentrations (0.5, 0.76, and 1.0 mg/ml) of 42,000 ● 1400 was obtained. Whole cell values for the number, Z, and $(Z + 1)$ average molecular weights were $M_n = 36{,}700$, $M_Z = 43{,}200$ and $M_{(Z+1)} = 44{,}300$, respectively, indicating little evidence for association–dissociation of the enzyme preparation. The molecular weight calculated from a calibration curve made up with standard protein markers in sodium dodecyl sulfate polyacrylamide gel electrophoresis gave an average molecular weight of 43,500 ± 1500 from three separate gels, in good agreement with the sedimentation equilibrium data.

Amino Acid Analyses. Table II presents the amino acid composition of the purified yeast protein kinase; values represent the averages obtained from three times of hydrolysis on two preparations of the enzyme. No amino sugar could be detected on the analyzer and no evidence could be obtained for nucleotides by uv spectrophotometry.

Stability. The enzyme can be exposed for a few hours to pH 5.8–9.5 at 30° without loss of activity but is unstable below pH 5.5. It is stable when stored at −20° in buffer A, but slowly loses activity at 4° (half-life ca. 40 days).

Substrate Specificity. Only the acidic phosphoproteins β_A- and α_{S1B1}-casein and phosvitin served as good phosphate acceptors for the yeast protein kinase; when present at 10 mg/ml, initial velocities of 1.05, 0.24, and 0.34 nmol of phosphate incorporated/min in buffer A (pH 7.5) at 30° were obtained, respectively. The following proteins were not phosphorylated by the purified enzyme: arginine- and lysine-rich histone, protamine; yeast phosphorylase *b*, phosphofructokinase, and pyruvate carboxylase; dogfish (*Squalus acanthias*) and rabbit muscle phosphorylase and phosphorylase kinase; a dogfish phosphate-acceptor protein related to parvalbumin (Blum et al., 1974), bovine serum albumin, and egg white lysozyme. Likewise, serine and polyserine (mol wt ca. 9700, Miles Laboratories) did not serve as phosphate acceptors. Yeast phosphorylase *b* was phosphorylated by early fractions of the kinase purification, e.g., through step 5 but not beyond that (see Table I). Obviously, these fractions must have contained a specific phosphorylase kinase that was eventually lost in the course of purification.

The reaction rates listed above should not be considered

Table II: Amino Acid Composition of Yeast Protein Kinase.[a]

Amino Acid	Residues/mol	Nearest Integer (moles/42,000 g of Protein)
Lysine	29.2 ± 1.3	29
Histidine	13.4 ± 0.6	13
Arginine	18.7 ± 0.6	19
Aspartic acid	41.6 ± 1.7	42
Threonine[b]	17.9 ± 0.6	18
Serine[b]	25.4 ± 1.4	25
Glutamic acid	41.9 ± 1.2	42
Proline	21.2 ± 1.6	21
Glycine	27.4 ± 0.5	27
Alanine	18.3 ± 0.2	18
Valine[c]	15.4 ± 0.5	15
Methionine	5.9 ± 0.2	6
Isoleucine[c]	20.4 ± 0.5	20
Leucine	33.2 ± 1.2	33
Tyrosine	13.6 ± 0.5	14
Phenylalanine	12.9 ± 0.3	13
Half-cystine[d]	3.0	3
Tryptophan[e]	5.7	6
Total residues		364

[a] Average of 24-, 48-, and 72-hr hydrolyses on two preparations of the enzyme. [b] Extrapolated to zero time of hydrolysis. [c] Calculated from 72-hr hydrolysis. [d] Determined as cysteic acid according to Hirs (1967). [e] Measured spectrophotometrically according to Bencze and Schmid (1957).

in absolute terms: casein and phosvitin introduced as substrates are already phosphorylated to different degrees; obviously, the rate of extent of phosphorylation will vary in relation to this phosphate "charge". As will be shown later, within wide limits, the rate of phosphate incorporation *decreases* as the number of susceptible sites (free seryl residues) *increases*.

Reverse Reaction. Using a brewer's yeast enzyme and phosvitin as substrate, Rabinowitz and Lipmann (1960) first noted that the reaction was reversible, i.e., that phosphate groups introduced into the protein could be transferred back to ADP to form ATP. The purified protein kinase described herein was therefore tested for its ability to catalyze this reverse reaction using as substrate both pure ^{32}P-labeled β_A-casein and a ^{32}P-labeled tryptic phosphopeptide (β_A-T1) derived from it; Figure 7 shows the pH dependence of both the forward and reverse reactions. As pointed out by Rabinowitz and Lipmann (1960) it is to be expected that the forward reaction would have a higher pH optimum than the reverse reaction, since protons are liberated in the first instance and consumed in the other. Indeed, the forward reaction showed a broad pH optimum around 7.5, while the reverse reaction displayed an optimum pH at or below pH 5.2, a value which could not be determined precisely since casein starts to precipitate under these conditions. Identification of [^{32}P]ATP as a reaction product is described below; neither AMP, UDP, CDP, nor GDP could serve as a phosphate acceptor.

Reversibility of the Kinase Reaction in the Presence of a Casein Phosphopeptide (β_A-T1). The 24-residue long phosphopeptide β_A-T1 obtained from a tryptic digestion of β_A-casein (50 mg in 10 ml of 0.1 *M* ammonium carbonate buffer, pH 8.0) was isolated according to Ribadeau-Dumas et al. (1971). The pure peptide was phosphorylated in the presence of 10 m*M* Mg^{2+} and 5 m*M* [γ-^{32}P]ATP; excess ATP was removed by passage through a 1 × 110 cm column of Sephadex G-15 in 1 *M* acetic acid. The resulting

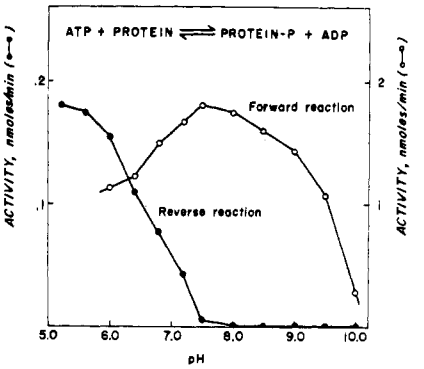

FIGURE 7: pH profiles of the forward and reverse reactions. The following buffers (0.1 *M*) were used: sodium acetate, pH 5.2–6.0; sodium morpholinopropanesulfonate, pH 6.0–7.2; Tris-HCl, pH 7.5–9.0; sodium glycinate, pH 9.0–10.0. For the forward reaction, 1% β_A-casein, 5 m*M* [γ-^{32}P]ATP (specific activity 1.3 × 10^7 cpm/μmol), and 10 m*M* Mg^{2+} was used. The reverse reaction was carried out in the presence of 1% ^{32}P-labeled β_A-casein (specific radioactivity of 2.9 × 10^6 cpm/μmol), 2 m*M* ADP, and 10 m*M* Mg^{2+}.

^{32}P-labeled phosphopeptide was free of nucleotides as determined by absorbance measurements at 257 nm. It was dissolved in 10 ml of 0.1 *M* ammonium formate (pH 5.5) and incubated with protein kinase, 10 m*M* Mg^{2+}, and 2 m*M* ADP for 1 hr at 30°, and rechromatographed on the same column. Material from the second peak representing the nucleotide fractions was analyzed on thin-layer cellulose sheets using the solvent system of Michelson (1959); both ADP and ATP were identified. Neither AMP nor P_i could be detected indicating that the protein kinase was free of myokinase, ATPase, or phosphatase activity. When the spots corresponding to AMP, ADP, and ATP were cut out and counted for ^{32}P radioactivity using the dioxane based scintillant given under Materials and Methods, 99.5% of the total radioactivity (22,770 cpm/10 μl) could be accounted for in the ATP spot and only 0.05 and 0.39% in the spots corresponding to AMP and ADP, respectively. These results demonstrate that phosphate groups can be indeed transferred to ADP from a relatively small peptide, e.g., that the intact tertiary structure of the protein substrate is not required.

Metal Ion Requirements and Salt Effects. The metal ion requirement of the purified enzyme was tested in both the forward and reverse reactions using β_A-casein as substrate and 2 m*M* salt; surprisingly, different behaviors were observed. In the forward reaction, maximum activity (taken as 100%) was observed with both Mg^{2+} and Mn^{2+}; Co^{2+}, Fe^{2+}, and Zn^{2+} displayed some activity (64, 16, and 8%, respectively) while Ca^{2+}, Ba^{2+}, Ni^{2+}, and Cu^{2+} were totally inactive. In the reverse direction, however, Mn^{2+} was five times less effective than Mg^{2+} while Fe^{2+} was totally ineffective.

Salts also affected both reactions differently when present at relatively high concentrations (100 m*M*). While NaF, sodium phosphate, and $MgCl_2$ strongly inhibited the forward and reverse reaction, most other salts inhibited the forward reaction only leaving the reverse reaction unaffected. Inhibition increased with the size of the anion (Cl < Br < I) and appeared to follow the Hofmeister series with respect to cation Mg^{2+} > Li > Na > K. It should be emphasized that these reactions were carried out at pH's close to their respective optima (7.5 and 6.0, respectively) and, therefore, that the values obtained are not directly comparable.

Table III: Kinetic Parameters of Protein Kinase Using β_A-Casein as Substrate.[a]

K_m(ATP)	0.2 m*M*
$\bar{K}$(ATP)	0.2 m*M*
K_m(β_A-casein)	0.166 m*M*
$\bar{K}$(β_A-casein)	0.146 m*M*
V_{max}	1.95 nmol/min

[a] Carried out in buffer A (pH 7.5) at 30°; Mg^{2+} was held constant at 10 m*M*.

Effectors of Yeast Protein Kinase. Cyclic 3′,5′-nucleotides were tested at several concentrations and at various stages of enzyme purification. No change in activity was observed in the presence of cyclic 3′,5′-AMP, -GMP, -UMP and -CMP, over a concentration range of 10^{-3}–10^{-6} *M* when either β_A-casein or histones were used as substrates. Furthermore, the enzyme was affected neither by the heat stable protein inhibitor which inactivates the rabbit muscle cAMP-dependent protein kinase (Walsh et al., 1971), nor by the regulatory subunit of rabbit muscle cAMP-dependent protein kinase (Reimann et al., 1971), using casein as substrate.

Kinetics. The kinetic parameters of the enzyme were determined using both β_A-casein and [γ-^{32}P]ATP as substrates. Initial rates were determined at three time points for each substrate concentration and the data analyzed according to Lineweaver and Burk (1936); the slopes and intercepts obtained were then reanalyzed by the procedure of Cleland (1967) to produce secondary plots from which the various parameters listed in Table III were derived. Essentially identical affinity constants were obtained when the concentration of both substrates was varied; these data suggest but do not prove that the reaction follows a random bi-bi mechanism in which the order of addition of the substrates is immaterial.

Site of Phosphorylation of β_A-Casein. β_A-Casein was phosphorylated exhaustively with [γ-^{32}P]ATP and the reaction terminated by precipitation of the substrate at pH 4.7. The suspension was centrifuged for 5 min at 5000*g* and the pellet dissolved in 0.1 *M* ammonium bicarbonate. This solution was extensively dialyzed against the same buffer, digested with trypsin, and chromatographed on Dowex 50-X2 (Figure 8) according to Ribadeau-Dumas et al. (1971). The radioactivity was totally recovered in the first two peaks, corresponding to the 24-residue long NH_2-terminal peptide β_A-T1 in peak 2, and to the same peptide lacking the NH_2-terminal arginyl residue in peak 1; both peptides share the same unique sequence -Glu-Ser(P)-Leu-Ser(P)-Ser(P)-Ser(P)-Glu-Glu- (see Ribadeau-Dumas et al., 1971).

Stoichiometry of the Phosphorylation Reaction. β_A- and α_{S1B}-caseins (mol wt 24,000 and 23,600, respectively) are both pure proteins in terms of their amino acid compositions and sequences (Mercier et al., 1971; Ribadeau-Dumas et al., 1972) but microheterogeneous in terms of their phosphate content. Phosphate analysis of β_A-casein yielded 4.45 ± 0.10 mol of phosphate/mol instead of 5, and the NH_2-terminal peptide β_A-T1, 3.40 ± 0.10 instead of 4, following exhaustive phosphorylation with protein kinase and [γ-^{32}P]ATP, β_A-casein incorporated an additional 0.58 ± 0.05 equiv of phosphate (0.27 ± 0.03 only for α_{S1B}-casein) and peptide β_A-T1 0.56 ± 0.06 mol of phosphate/mol, corresponding in both cases to a complete phosphorylation of all susceptible groups. These data confirm that isolated β_A-casein and, presumably, α_{S1B}-casein, are heterogeneous in terms of their phosphate content.

Phosphorylation of Partially Dephosphorylated β_A-Casein and Phosphopeptide β_A-T1. β_A-Casein and the phosphopeptide β_A-T1 were partially dephosphorylated as indicated under Materials and Methods. Figure 9 illustrates the rate of phosphate incorporation for β_A-casein at different levels of phosphate depletion. As can be seen, it decreases drastically with the phosphate content of β_A-casein; the initial increase is misleading and only reflects the appearance of free seryl residues, i.e., the rate of phosphorylation would necessarily fall to zero if the peptide were fully phosphorylated.

In an attempt to further clarify the distribution and behavior of seryl-P residues in the molecule, the susceptibility of peptide β_A-T1 to dephosphorylation and rephosphorylation was investigated. Since it contains only 3.4 phosphate groups/mol out of a total of 4 expected (85% phosphorylation) it must represent a mixture of peptides with identical sequences but differently charged with phosphate groups. Two alternative hypotheses could be advanced to account for this deficiency. On the one hand, one could assume that a single seryl residue is exceptionally susceptible to dephosphorylation; in that case, one would be dealing exclusively with a mixture of two species, namely, 40% of the fully phosphorylated peptide (P_4) and 60% of the species (P_3) with only three phosphate groups. On the other hand, dephosphorylation could be entirely random; a similar situation was considered for the dephosphorylation of phosphorylase *a*, a tetramer also containing four phosphate groups/molecule (Fischer et al., 1968). Using the same mode of analysis, one can calculate that the peptide β_A-T1 would then contain approximately 55% of the fully phosphorylated species (P_4), 35% of P_3, 8% of P_2, 2% of P_1, and essentially no P_0.

To investigate this point, a paper electrophoresis system utilizing the pH 3.5 solvent system of Ryle et al. (1955) for 90 min at 2500 V was devised for the separation of the variously phosphorylated peptides. R_f values (relative to the fastest migrating species P_4 taken as R_f = 1.0) were: 0.72, 0.44, 0.15, and −0.08 for P_3, P_2, P_1, and P_0, respectively.

The peptide mixture (1.25 μmol/ml) was subjected to the action of the protein kinase in the presence of [γ-^{32}P]ATP in 0.1 *M* ammonium bicarbonate (pH 8.0) for 20 min; this time period was chosen arbitrarily since, of course, one would get a single, fully phosphorylated species (P_4) containing all the radioactivity if phosphorylation were allowed to go to completion; 10 μl was then removed, spotted on paper, and subjected to electrophoresis as described above. After spraying with ninhydrin, spots corresponding to the various phosphorylated intermediates P_4, P_3, P_2, and P_1 were cut out and counted (see Materials and Methods). The distribution of ^{32}P as a function of the phosphate level in β_A-T1 is listed in Table IV. Since P_4 is already fully phosphorylated, phosphate incorporation can only occur in peptides P_3, P_2, P_1, and P_0, present in peptide β_A-T1 in the relative proportion of ca. 77, 18, 4, and 1%, respectively. The data in Table IV (first line) indicate that phosphorylation occurred to the relative extent of ca. 65.5, 17, 10, and 7%, respectively. Despite the limitations of this approach, the experiment clearly shows that (a) the original peptide β_A-T1 consists of a mixture of all possible phosphorylated intermediates, and, therefore, that phosphorylation or dephosphorylation occurs randomly, not at a single site; (b)

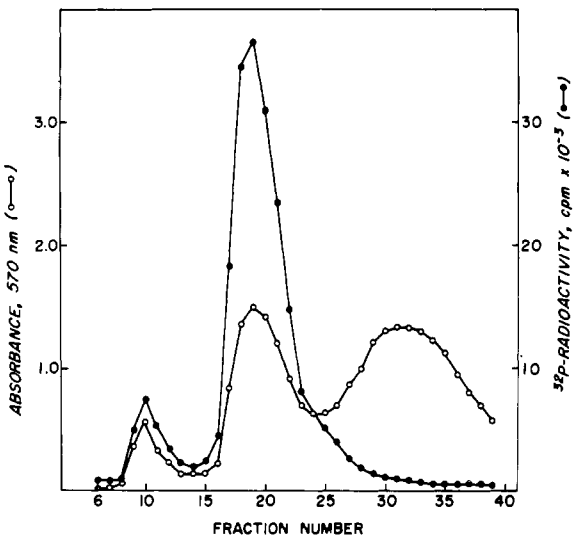

FIGURE 8: Dowex 50-X2 chromatography of a tryptic digest of ^{32}P-labeled β_A-casein. The column (1.0 × 100 cm) was run at 37° in 0.2 *M* pyridine acetate buffer (pH 3.1); elution was carried out in the same buffer without applying a gradient. The flow rate was 20 ml/hr; 3-ml fractions were collected. Ninhydrin reactions were determined after alkaline hydrolysis according to Hirs (1967) and 0.1-ml aliquots were counted for ^{32}P radioactivity.

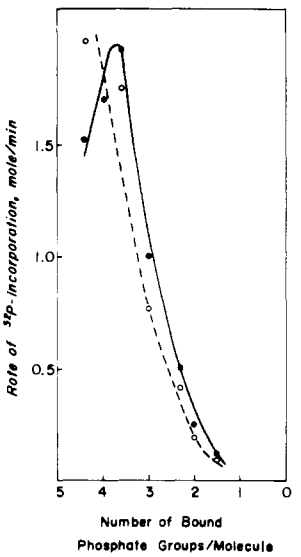

FIGURE 9: Rate of ^{32}P incorporation as a function of the number of phosphate groups in the β_A-casein. β_A-Casein (5 mg/ml) was dephosphorylated to various extents by almond acid phosphatase as indicated under Materials and Methods; rephosphorylated in buffer A (pH 7.5) at 30° in the presence of protein kinase, 5 m*M* [γ-^{32}P]ATP, and 10 m*M* Mg^{2+}. Aliquots were removed after 2, 4, and 6 min and measured for bound radioactivity for determination of initial rates. The solid curve represents the direct values obtained; the broken line represents an attempt to correct these data for the changes in the concentration of the *P*-accepting groups (i.e., free seryl-residues) assuming no differences in K_m for the various phosphorylated species (see text).

almond acid phosphatase also removes phosphate randomly; and (c) the rate of phosphorylation decreases precipitously as the phosphate "charge" of the peptide decreases.

Discussion

The specific activity of the purified kinase obtained here in homogeneous form after 17,000-fold purification (ca. 10 μmol of phosphate transferred per min per mg of protein) compares favorably with that of most other purified protein phosphokinases which varies from a few nanomoles to 30–35 μmol of phosphate transferred per min per mg of protein. In any event, these values have little absolute meaning since the reaction rates vary considerably with the nature of the substrate, if not with its extent of phosphorylation as described herein. An added complication is that, of course, casein is not the natural substrate for the yeast kinase and no information has been obtained as yet on the nature of the endogenous protein serving as the physiological phosphate acceptor. At first it was thought that phosphorylase might serve this function, but the purified kinase obtained is totally inactive on this substrate. Yeast must therefore contain another phosphokinase specific toward phosphorylase that has yet to be isolated and characterized.

The difference in substrate specificity among protein kinases is also poorly understood, though it might become clearer as more information accumulates on the amino acid sequence of the segments that are phosphorylated. While some enzymes have rather restricted action patterns (e.g., phosphorylase kinase which acts on two or three proteins at most), others seem to enjoy a much broader specificity. A distinction might be made between those enzymes which catalyze the phosphorylation of acidic as opposed to basic substrates (e.g., casein and phosvitin vs. protamines and histones). The kinase described herein, just like the brain enzyme of Rabinowitz and Lipmann (1960), has a high isoelectric point and seems to act exclusively on acidic substrates; it is inactive on protamines and histones. By contrast, the yeast cAMP-dependent protein kinase of Takai et

Table IV: Phosphorylation of Partially Dephosphorylated Phosphopeptide β_A-T1.

Extent of Dephosphorylation (%)	mol of Phosphate Remaining per mol of Peptide	^{32}P Incorporated/mol of Peptide in Intermediate Species				Total mol of Phosphate Incorporated per mol of Peptide	% Rephosphorylation in 20 min
		P_4	P_3	P_2	P_1		
15[a]	3.4	0.38 (65.5)[b]	0.10 (17.2)	0.06 (10.3)	0.04 (7)	0.58 (100)	99.5
25	3.0	0.29	0.20	0.07	0.06	0.62	90.5
34	2.64	0.24	0.40	0.16	0.06	0.86	87.5
59	1.64	0.09	0.16	0.23	0.13	0.61	56
71	1.16	0.07	0.09	0.20	0.17	0.53	42
94	0.24	0.04	0.04	0.05	0.19	0.32	14

[a] Original peptide β_A-T1 as isolated. [b] Numbers in parentheses indicate percentage of counts found in each peptide; since peptide P_4 is already fully phosphorylated, all counts found in this peptide can only result from the phosphorylation of P_3, resulting in a $P_3 \rightarrow P_4$ conversion.

al. (1974) phosphorylates protamines and histones but neither casein nor phosvitin.

Yeast contains a relatively high ratio of threonine-P to serine-P (1:8 as compared to ca. 1:20 for rat liver or muscle, or 1:38 for *Escherichia coli,* Rask et al., 1970). While only seryl residues were shown to be phosphorylated by the purified kinase, the possibility that threonyl side chains might also become esterified in other substrates has not been excluded. By contrast, a single threonyl residue is modified by yeast phosphorylase kinase during the phosphorylase *b* to *a* conversion (Lerch and Fischer, 1975) and the yeast cAMP-dependent protein kinase phosphorylates both seryl and threonyl side chains.

Protein kinases can also be distinguished on the basis of their regulatory properties. The enzyme described here is not affected by cyclic nucleotides either in crude extracts obtained under various conditions or in purified form; it consists of a single catalytically active subunit and is not inhibited either by the regulatory subunit of mammalian cAMP-dependent protein kinases (Brostrom et al., 1970; Gill and Garren, 1970) or the "heat-stable" inhibitor described by Walsh et al. (1971). Likewise, neither yeast phosphorylase kinase nor calf-brain phosvitin kinase (Walinder, 1973), which both act on acidic regions of proteins, appears to be regulated by cyclic nucleotides. The latter is said to be associated with a second protein that becomes phosphorylated during the enzymatic reaction, but no regulatory function could be ascribed to this component. Therefore, one might ask what controls the catalytic activity of these enzymes. By contrast, most protein kinases of higher vertebrates that are involved in the metabolic regulation of enzymes appear to be under direct or indirect hormonal regulation; they all have oligomeric structures and some of their subunits serve to modulate their catalytic activity. A cAMP-binding protein of molecular weight 24,000 was isolated from several yeast strains (Sy and Richter, 1972); while no function could be ascribed to this protein at first, it seems very likely to be identical with the regulatory subunit of the yeast cAMP-dependent protein kinase described by Takai et al. (1974); the enzyme is inhibited by the regulatory subunit of mammalian protein kinases.

Surprisingly, however, the catalytic subunits of many of these enzymes seem to have similar molecular weights, whether they are cAMP dependent or not (e.g., 42-, 46-, and 41 $\times 10^3$ for the cAMP-independent protein kinase from yeast (this manuscript), dogfish (Blum et al., 1974), and calf brain (Walinder, 1973) and 40-, 42-, 42-, and 49 $\times 10^3$ for the cAMP-dependent enzymes from beef brain (Miyamoto et al., 1973), beef heart (Rubin et al., 1972), rabbit adipose tissue (Corbin et al., 1972a), and rabbit muscle (Corbin et al., 1972b), respectively.

The factors determining the reversibility of the kinase reaction depend, of course, upon the nature of the substrate, not that of the enzyme. As pointed out by Rabinowitz and Lipmann (1960), the seryl-P groups in phosvitin or casein must be of rather high energy, in contrast to phosphoserine itself; the same must be true for the seryl-P residues in most other proteins for which the phosphorylation reaction is essentially irreversible. Data presented here indicate that the "high-energy" character of seryl-P groups in casein depends solely on the primary structure of the substrate, not on its tertiary conformation: reversibility could be just as well demonstrated when the amino-terminal peptide β_A-T1 was used as substrate in place of the intact protein. In determining the sequence of α- and β-casein, Mercier et al. (1971) have pointed out that each phosphoseryl group is flanked by another negatively charged side chain one residue removed: . . . Ser(P)-X-Y . . . in which Y represents an aspartyl, glutamyl, or another seryl-P residue. Presumably, charge repulsion provides for much of the energy needed for the formation of ATP. While two negative charges 4 Å apart would produce an electrostatic repulsion of ca. 0.5 kcal/mol in 10 m*M* salt solution, in the sequence: . . . Glu-Ser(P)-Leu-Ser(P)-Ser(P)-Ser(P)-Glu-Glu . . . found in casein and peptide β_A/T1, one is dealing with a cluster of approximately ten negative charges at neutrality. Assuming very conservatively that these were distributed within a 15-Å radius, they would generate an internal electrostatic repulsion approaching 10 kcal/mol (Tanford, 1960). To this should be added the energy for a helix–coil type of transition if one assumes that the proximity of adjacent negative charges maintains the polypeptide chain in an extended conformation and that removal of some of the charges allows the molecule to collapse into a more compact structure. Ample physicochemical evidence has been presented for such a transition of phosvitin when the negative charges are cancelled (Timasheff and Townend, 1967; Grizzuti and Perlmann, 1970). The conversion from an extended structure in which the seryl-P residues are exposed into a more organized conformation in which these groups are masked would explain why the susceptibility of casein to phosphorylation falls drastically as the number of phosphoryl groups diminishes. As a consequence, one might expect that a rather specific phosphokinase is involved in the phosphorylation of newly synthesized casein molecules. Such a dephosphoprotein was shown to exist in the mammary gland (Turkington and Topper, 1966) and an enzyme was described in the Golgi apparatus of lactating animals that phosphorylates dephospho α_{S1}-casein four times faster than the normal protein (Bingham et al., 1972).

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References

Babul, J., and Stellwagen, E. (1969), *Anal. Biochem. 28,* 216.

Bencze, W. L., and Schmid, K. (1957), *Anal. Chem. 29,* 1193.

Bingham, E. W., Farrell, H. M., Jr., and Basch, J. J. (1972), *J. Biol. Chem. 247,* 8193.

Blum, H. E., Pocinwong, S., and Fischer, E. H. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 2198.

Bray, G. A. (1960), *Anal. Biochem. 1,* 279.

Brostrom, M. A., Reimann, E. M., Walsh, D. A., and Krebs, E. G. (1970), *Adv. Enzyme Regul. 8,* 191.

Clark, R. C. (1973), *Biochim. Biophys. Acta 310,* 174.

Cleland, W. W. (1967), *Annu. Rev. Biochem. 36,* 77.

Cohen, P. (1973), *Eur. J. Biochem. 34,* 1.

Corbin, J. D., Brostrom, C. O., Alexander, R., and Krebs, E. G. (1972a), *J. Biol. Chem. 247,* 3736.

Corbin, J. D., Brostrom, C. O., King, C. A., and Krebs, E. G. (1972b), *J. Biol. Chem. 247,* 7790.

Fischer, E. H., Hurd, S. S., Koh, P., Seery, V. L., and Teller, D. C. (1968), *in* Control of Glycogen Metabolism,

Whelan, W. J., Ed., New York, N.Y., Academic Press, p 19.
Fischer, E. H., and Krebs, E. G. (1958), *J. Biol. Chem. 231,* 65.
Fiske, C. H., and Subbarow, Y. (1925), *J. Biol. Chem. 66,* 375.
Fosset, M., Muir, L. W. Nielson, L. D., and Fischer, E. H. (1971), *Biochemistry 10,* 4105.
Gill, G. N., and Garren, L. D. (1970), *Biochem. Biophys. Res. Commun. 39,* 335.
Glynn, I. M., and Chappell, J. B. (1964), *Biochem. J. 90,* 147.
Grizzuti, K., and Perlmann, G. E. (1970), *J. Biol. Chem. 245,* 2573.
Hirs, C. H. W. (1967), *Methods Enzymol. 11,* 469.
Hsiu, J., Fischer, E. H., and Stein, E. A. (1964), *Biochemistry 3,* 61.
Kiddy, C. A., Thompson, M. P., Johnston, J. O., and Pepper, L. (1963), *J. Dairy Sci. 46,* 626.
Krebs, E. G. (1972), *Curr. Top. Cell. Regul. 6,* 99.
Langan, T. A. (1967), in Regulation of Nucleic Acid and Protein Biosynthesis, Konigsberger, V. V., and Bosch, L., Ed., Amsterdam, Elsevier, p 233.
Lerch, K., and Fischer, E. H. (1975), *Biochemistry,* preceding paper in this issue.
Lineweaver, H., and Burk, D. (1936), *J. Am. Chem. Soc. 56,* 658.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 254.
Manson, W., and Annan, W. D. (1971), *Arch. Biochem. Biophys. 145,* 16.
Mercier, J.-C., Grosclaude, F., and Ribadeau-Dumas, B. (1971), *Eur. J. Biochem. 23,* 41.
Mercier, J.-C., Maubois, J. L., Poznanski, S., and Ribadeau-Dumas, B. (1968), *Bull. Soc. Chim. Biol. 50,* 521.
Michelson, A. M. (1959), *J. Chem. Soc.,* 1371.
Miyamoto, E., Petzold, G. L., Kuo, J. F., and Greengard, P. (1973), *J. Biol. Chem. 248,* 179.
Moore, S., and Stein, W. H. (1963), *Methods Enzymol. 6,* 819.
Rabinowitz, M., and Lipmann, F. (1960), *J. Biol. Chem. 235,* 1043.
Rask, L., Walinder, O., Zetterquist, O., and Engstrom, L. (1970), *Biochim. Biophys. Acta 221,* 107.
Reimann, E. M., Walsh, D. A., and Krebs, E. G. (1971), *J. Biol. Chem. 246,* 1986.
Ribadeau-Dumas, B., Brignon, G., Grosclaude, F., and Mercier, J.-C. (1971), *Eur. J. Biochem. 20,* 264.
Rodbard, D., and Chrambach, A. (1972), *Anal. Biochem. 40,* 95.
Rubin, C. S., Ehrlichman, J., and Rosen, O. M. (1972), *J. Biol. Chem. 247,* 36.
Ryle, A. P., Sanger, F., Smith, L. F., and Kitai, R. (1955), *Biochem. J. 60,* 541.
Svedberg, T., and Pederson, K. O. (1940), The Ultracentrifuge, London, Oxford University.
Sy, J., and Richter, D. (1972), *Biochemistry 11,* 2784.
Takai, Y., Yamamura, H., and Nishizuka, Y. (1974), *J. Biol. Chem. 249,* 530.
Takeda, M., Yamamura, H., and Ohga, Y. (1971), *Biochem. Biophys. Res. Commun. 42,* 103.
Tanford, C. (1960), Physical Chemistry of Macromolecules, New York, N.Y., Wiley, p 457.
Teller, D. C., Horbett, T. A., Richards, E. G., and Schachman, H. K. (1969), *Ann. N.Y. Acad. Sci. 164,* 66.
Timasheff, S. N., and Townend, R. (1967), *J. Biol. Chem. 242,* 2290.
Turkington, R. W., and Topper, Y. J. (1966), *Biochim. Biophys. Acta 127,* 366.
Walinder, O. (1973), *Biochim. Biophys. Acta 293,* 140.
Walsh, D. A., Ashby, C. D., Gonzalez, C., Calkins, D., Fischer, E. H., and Krebs, E. G. (1971), *J. Biol. Chem. 246,* 1977.
Walsh, K. A., and Brown, J. R. (1962), *Biochim. Biophys. Acta 58,* 596.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406.
Yphantis, D. A. (1964), *Biochemistry 3,* 297.