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Reversible Phosphorylation of T-Substrate by Wheat Germ, Human Erythrocyte, and Rabbit Skeletal Muscle Protein Kinases†

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ABSTRACT: The reversibility of the reactions catalyzed by the wheat germ kinase and the cyclic AMP independent protein kinases isolated from human erythrocytes (casein kinases A and G) and rabbit skeletal muscle (casein kinases I and II) has been investigated. The reverse reaction requires ADP, Mg^{2+}, phosphoprotein, and kinase and results in the formation of ATP from the phosphoprotein and ADP. The requirement for ADP in the wheat germ kinase and casein kinases II and G catalyzed reactions appears to be nonspecific. These kinases can also utilize GDP, IDP, and UDP as phosphoryl acceptors. Studies with the wheat germ protein T-substrate indicate that the phosphorylation of this protein substrate by the kinases is fully reversible. By contrast, the phosphorylation of phosvitin and casein is only partially reversible. Since the T-substrate is found to contain multiple phosphorylation sites and can serve as phosphoryl acceptor for the various kinases, the specificity of the phosphorylation of the substrate by the kinases is examined by way of the reverse reaction. The wheat germ kinase, casein kinase G, and casein kinase II appear to phosphorylate the same sites on the T-substrate as they are capable of completely dephosphorylating each other's ^{32}P-T-substrate. Each of these kinases can catalyze the incorporation of 12 mol of ^{32}P/48 000 g of T-substrate. In contrast, casein kinases A and I can incorporate only 6 mol of ^{32}P/48 000 g of T-substrate. Studies on the reverse reactions suggest that these phosphorylation sites may be the same for both enzymes. It is of interest to note that the six sites phosphorylated by casein kinases A and I may be among those recognized by the wheat germ kinase and casein kinases G and II, as these kinases can completely dephosphorylate these sites via the reverse reaction.

We have recently reported the isolation of a cyclic AMP independent protein kinase from wheat germ extract (Yan & Tao, 1982a). This represents one of a few kinases from plant systems that has been purified to homogeneity. The enzyme catalyzes the phosphorylation of casein and phosvitin using either ATP or GTP as a phosphoryl donor. An endogenous phosphoryl acceptor, termed T-substrate, of the kinase has also been identified and purified. This endogenous substrate contains a large number of phosphorylation sites and is by far

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the best substrate for the wheat germ kinase as well as several other cyclic AMP independent protein kinases isolated from human erythrocytes and rabbit skeletal muscle (Yan & Tao, 1982b).

In this study, we examine the reversibility of the reactions catalyzed by the wheat germ kinase and also by similar kinases isolated from human erythrocytes and rabbit skeletal muscle. Our results indicate that the phosphorylation of T-substrate by these kinases is fully reversible. Evidence is presented to suggest that the various kinases may recognize the same phosphorylation sites on the T-substrate.

Experimental Procedures

Materials. Wheat germ was purchased from Dixie Portland Co., Chicago. [γ-^{32}P]ATP was obtained from Amersham Corp., casein from Chemical Dynamics Corp., and histone IIA from Sigma Chemical Corp.

Protein Kinase Assays. The casein kinases were assayed as described earlier (Yan & Tao, 1982a). The reaction mixture (0.2 mL) contained 50 mM tris(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl),[1] pH 7.5, 5 mM $MgCl_2$, 1.5 mg/mL casein, 0.2 mM [γ-^{32}P]ATP (15–30 cpm/pmol), and enzyme protein. The incubation was conducted at 37 °C for 5 min, unless indicated otherwise, and the reaction terminated by the addition of about 2 mL of 10% trichloroacetic acid. The radioactivity incorporated into casein was determined as described by Tao et al. (1980).

The catalytic subunit of the cyclic AMP dependent protein kinase was assayed as described above except with histone IIA as substrate.

One unit of kinase activity was defined as that amount of enzyme which catalyzed the incorporation of 1 nmol of phosphate into casein or histone per min. Protein concentrations were determined by the procedure of Bradford (1976) and also by that of Lowry et al. (1951) with crystalline bovine serum albumin as standard.

Preparation of Protein Kinases. Homogeneous preparation of the wheat germ kinase was obtained as described earlier (Yan & Tao, 1982a). The casein kinases from rabbit skeletal muscle were isolated according to the method of Itarte et al. (1977) with minor modifications. Glycerol was omitted from all the buffers used in the various purification steps, and the initial treatment of the muscle extract with glycogen was not performed. The absence of glycerol in the buffers led to the precipitation of a large amount of contaminating proteins during purification. These precipitates were removed by centrifugation. The casein kinases eluted from the phosphocellulose column at KCl concentrations of 0.4 M (casein kinase I) and 0.5 M (casein kinase II) were further purified by Sephacryl S-300 gel filtration. Analyses by sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel electrophoresis indicate that approximately 85% of the proteins in casein kinase I were attributed to an M_r = 31 000 component, whereas in kinase II, greater than 90% of the proteins were distributed in two bands of M_r 43 000 and 25 000.

The procedure employed for the purification of the human erythrocyte cytosolic casein kinases was adopted in part from that of Simkowski & Tao (1980). The membrane-free crude hemolysate was directly adsorbed to a DEAE-cellulose column. The column was eluted with a 0–0.4 M KCl gradient. As shown by Simkowski & Tao (1980), two casein kinase activities were resolved from the column. The kinase activity eluted at about 0.15 M KCl was designated as casein kinase A, whereas that eluted at 0.2 M was designated as casein kinase G. Kinase A was futher purified by phosphocellulose chromatography, Sephacryl S-300 gel filtration, and DEAE-cellulose chromatography. Kinase G was also further purified by Sephacryl S-300 gel filtration and by T-substrate-substituted Sepharose-4B affinity column chromatography (Yan & Tao, 1982a). Although these two kinases were purified extensively, the enzyme preparations still contained a few minor contaminants as determined by $NaDodSO_4$–polyacrylamide gel electrophoresis.

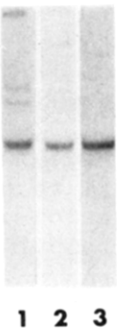

FIGURE 1: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of T-substrate before (gel 1) and after (gel 2) isoelectrofocusing. In order to verify that the component purified represented T-substrate, one of the samples applied to the gel (gel 2) was phosphorylated with [γ-^{32}P]ATP (250 cpm/pmol) in the presence of 0.05 μg of wheat germ kinase for 5 min under the conditions described earlier (Yan & Tao, 1982b). Electrophoresis was conducted in a 5.6% gel slab according to the procedure of Tao et al. (1980). The radioautogram (gel 3) was prepared by exposing (for 1 h) a Kodak No Screen film to the dried gel. A Dupont Lightning Plus intensifying screen was used during exposure in order to shorten the exposure time. Approximately 10 μg of protein was applied to gel 1 and 7 μg to gel 2.

The catalytic subunit of the cyclic AMP dependent protein kinase used in this study was prepared by the method (method I) described by Bechtel et al. (1977).

Preparation of T-Substrate. The T-substrate obtained as described previously (Yan & Tao, 1982b) was isoelectrofocused in a pH 3–10 gradient. The protein precipitate which focused at pH 7.5 was collected by centrifugation and washed twice with a buffer (buffer A) containing 20 mM Tris-HCl, pH 7.5, 15 mM mercaptoethanol, 1 mM EDTA, and 0.05 mM phenylmethanesulfonyl fluoride (PMSF). The pellet was dissolved in buffer A containing 1.0 M KCl, and the residual ampholyte was removed by Sephadex G-50 (1 × 27 cm column) gel filtration followed by phosphocellulose column (1.2 × 4 cm) chromatography. The final protein preparation was analyzed for purity by $NaDodSO_4$–polyacrylamide gel electrophoresis. Figure 1 shows that the preparation contains a single protein-staining component which can be phosphorylated by the wheat germ kinase.

The highly purified T-substrate was found to contain approximately 2.5 mol of alkali-labile protein-bound phosphate per 48 000 g of protein. An analysis of the extent of phosphorylation of the T-substrate by the various kinases indicates that a maximum of 12 mol of phosphate is incorporated per 48 000 g of protein in reactions catalyzed singly by the wheat germ kinase, casein kinase II, or casein kinase G or by a combination of these kinases. The two other enzymes, casein kinase I and casein kinase A, used in this study catalyzed the incorporation of about 6 mol of phosphate per 48 000 g of T-substrate. It should also be noted that a reaction mixture containing, for example, both the wheat germ kinase and casein kinase A did not result in phosphorylation of the T-substrate

[1] Abbreviations: PMSF, phenylmethanesulfonyl fluoride; Mes, 2-(*N*-morpholino)ethanesulfonate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

exceeding the 12 mol of phosphate/48 000 g observed with the wheat germ kinase alone.

Preparation of ^{32}P-Labeled Substrates. The labeling of the T-substrate (0.75–1.5 mg) by either wheat germ kinase (6 μg) or casein kinase II (3 units) was conducted in a reaction mixture (0.8 mL) containing 50 mM Tris-HCl, pH 7.5, 0.5 mM [γ-^{32}P]ATP (700–1000 cpm/pmol), and 5 mM $MgCl_2$. The phosphorylation by casein kinase I (2 units) was conducted under the same conditions except that the Tris-HCl buffer was replaced with 2-(*N*-morpholino)ethanesulfonate (Mes) buffer, pH 6.0. Incubation of the reaction mixtures was conducted at 37 °C for 60 min. The ^{32}P-labeled T-substrate was separated from the kinase and unreacted [γ-^{32}P]ATP by gel filtration on a Sephacryl S-200 column (1 × 110 cm). The column was eluted with buffer A containing 0.5 M KCl. Because casein kinase II failed to resolve completely from the ^{32}P-T-substrate on the Sephacryl S-200 column, the ^{32}P-T-substrate was further treated with phosphocellulose in the presence of 0.3 M KCl. The kinase was adsorbed to the phosphocellulose which was then removed by centrifugation.

^{32}P-Casein and ^{32}P-phosvitin were also prepared as described above except that the time of incubation was for 20 h. The unreacted [γ-^{32}P]ATP was removed by gel filtration on a Sephadex G-50 column (1 × 50 cm) and the kinase by adsorption to phosphocellulose.

The resultant ^{32}P-labeled protein preparations were free of [γ-^{32}P]ATP and of any kinase activity. The ^{32}P-T-substrate was stored in buffer A containing 0.1 M KCl at 0–4 °C, whereas ^{32}P-casein and ^{32}P-phosvitin were stored in buffer A at −20 °C. These ^{32}P-labeled protein substrates were used within 2 weeks.

Assays for the Reverse Reaction. The reverse reaction catalyzed by the kinases was assayed by measuring the release of ^{32}P from the ^{32}P-labeled proteins into the trichloroacetic acid soluble fraction. The assay mixture (25 μL) contained 5 mM $MgCl_2$, 5 mM ADP, ^{32}P-labeled protein (as indicated in the table or figure legends), protein kinase, and either 50 mM Mes, pH 5.8, buffer (when wheat germ kinase, casein kinase I, casein kinase A, or the cyclic AMP dependent protein kinase was used) or imidazole hydrochloride, pH 7.0, buffer (when casein kinase II or G was used). The incubation was carried out at 37 °C for an appropriate time interval and the reaction terminated by adding 15 μL of 50% trichloroacetic acid and 10 μL of 1 mg/mL bovine serum albumin. The mixture was kept in an ice–water bath for 5 min and centrifuged. An aliquot of 35 μL was withdrawn from the supernatant for radioactivity determination.

Determination of Equilibrium Constants. The equilibrium constants (K_{eq}) of the wheat germ kinase catalyzed reaction are calculated on the basis of the following simplified equation:

$$K_{eq} = \frac{[\text{ATP}][\text{T-substrate}]}{[\text{ADP}][^{32}\text{P-T-substrate}]}$$

The equation is written for the reverse reaction with the assumption that all the phosphorylation sites on the T-substrate are equivalent.

Determination of K_{eq} was conducted at two different pH values, 5.8 and 7.5, corresponding to the pH optima for the reverse and the forward reactions, respectively. The reaction mixture (0.5 mL) contained 20 μg/mL wheat germ kinase, 50 mM either of Tris-HCl, pH 7.5, or of Mes, pH 5.8, 5 mM $MgCl_2$, and varying concentrations of [γ-^{32}P]ATP (sp act. = 120 cpm/pmol) and T-substrate for the forward reaction (at pH 7.5) or ADP and ^{32}P-T-substrate (12 mol of ^{32}P/48 000 g of protein) for the reverse reaction (at pH 5.8). Incubation was conducted at 37 °C for a period of either 1 h (pH 7.5) or 4 h (pH 5.8). At appropriate time intervals, aliquots were withdrawn, and the concentrations of the various reaction components were determined. The concentration of ADP was measured according to the procedure of Lowry & Passonneau (1972), whereas that of [γ-^{32}P]ATP was determined by measuring the amount of the radioactivity present in the trichloroacetic acid soluble fraction as described under Assays for the Reverse Reaction. [^{32}P-T-substrate] was determined as in the kinase assays described above.

Table I: Incubation Components of the Dephosphorylation Reactions[a]

	^{32}P released (cpm)				
reaction mixture	WGK	CK-I	CK-II	CK-A	CK-G
complete	58 000	18 200	32 700	12 700	23 600
−ADP	530	690	720	530	530
−Mg^{2+}	500	670	720	600	550
−kinase	470	500	500	530	510
zero-time control	510	700	500	550	530

[a] The complete reaction mixture (25 μL) contained 5 mM ADP, 5 mM $MgCl_2$, 50 μg/mL ^{32}P-T-substrate (12 mol of ^{32}P/48 000 g), 50 mM buffer, and 0.05 unit of the wheat germ kinase (WGK) or 0.1 unit of casein kinase I (CK-I), II (CK-II), A (CK-A), or G (CK-G). The dephosphorylation reactions catalyzed by WGK, CK-I, and CK-A were conducted in Mes buffer, pH 5.8, whereas those catalyzed by CK-II and CK-G were conducted in imidazole hydrochloride buffer, pH 7.0, as described in the text.

Results

General Requirement of the Kinase-Catalyzed Reverse Reaction. The reversibility of the reaction catalyzed by the wheat germ kinase was examined by using ^{32}P-T-substrate. As shown in Table I, the liberation of radioactivity from ^{32}P-labeled T-substrate into the trichloroacetic acid soluble fraction requires the presence of the wheat germ kinase, ADP, and Mg^{2+}. In the absence of any of these components, no significant radioactivity over the background level was released. The data suggest that the ^{32}P released from ^{32}P-T-substrate probably resulted from the transfer of the phosphate to ADP by way of the reverse reaction catalyzed by the wheat germ kinase. The rate of phosphoryl transfer was dependent upon the kinase concentration (data not shown).

We have similarly examined the dephosphorylation reactions catalyzed by casein kinases I, II, A, and G. Our results show that all these kinases are capable of causing the release of ^{32}P from ^{32}P-T-substrate. Likewise, the reactions required the presence of both ADP and Mg^{2+} (Table I).

Effect of pH on the Phosphorylation and Dephosphorylation of T-Substrate. Figure 2 shows the pH profiles of the reverse (dephosphorylation) as well as the forward (phosphorylation) reactions catalyzed by the wheat germ kinase and casein kinases I and II. For the wheat germ kinase, the dephosphorylation reaction was optimum at about pH 5.8 (Figure 2A). The forward reaction, on the other hand, exhibited a broad pH optimum spanning from 7 to 8.5.

In the case of casein kinases I and II, their pH profiles for the forward and reverse reactions appeared to differ only slightly. The pH profiles of the reverse reaction for both kinases were slightly on the acidic side as compared to those of the forward reaction. The reverse reaction pH optima of casein kinases I (Figure 2C) and II (Figure 2B) are 6 and 7, respectively. Their corresponding forward reaction pH optima are higher by only about half a pH unit or less. The pH optima exhibited by kinases A and G are identical with those of kinases I and II, respectively (data not shown).

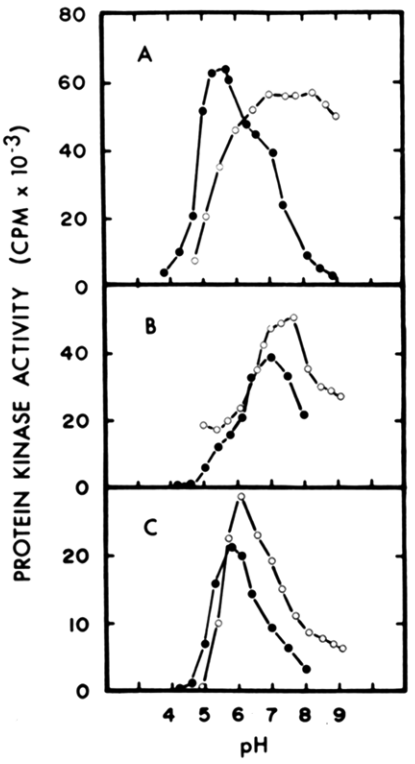

FIGURE 2: pH–activity profiles of the reverse (●) and forward (○) reactions catalyzed by the wheat germ kinase (A) and casein kinases I (C) and II (B). The ^{32}P-T-substrate (50 μg/mL) used in the reverse reactions contained about 10 mol of ^{32}P/48 000 g of T-substrate. In (A), the ^{32}P incorporated into T-substrate had a specific activity of 630 cpm/pmol and in (B) and (C), 750 cpm/pmol. The incubation for the reverse reactions was for 15 min. The forward reactions were conducted for 5 min in the presence of 0.2 mg/mL T-substrate and 0.2 mM [γ-^{32}P]ATP. The specific activity of [γ-^{32}P]ATP used in (A) was 34 cpm/pmol and in (B) and (C), 40 cpm/pmol. The buffers used were acetate (pH 3.8–5.2), Mes (pH 5.5–6.5), imidazole (pH 6.5–7.5), and Tris-HCl (pH 7.5–9.0).

Table II: Nucleotide Substrate Specificity of the Dephosphorylation Reactions[a]

	^{32}P released (pmol/min)				
nucleotide	WGK	CK-II	CK-G	CK-I	CK-A
ADP	11.4	3.6	2.6	2.0	1.4
GDP	8.6	2.9	2.0	0.1	0.1
IDP	7.2	2.2	1.2	0	0
UDP	6.1	2.1	1.1	0	0
CDP	0	0	0	0	0
NMP	0	0	0	0	0

[a] The dephosphorylation reactions were conducted as described under Experimental Procedures except in the presence of different nucleotides. The ^{32}P-T-substrate (56 μg/mL) was derived from phosphorylation of the protein by the wheat germ kinase to the extent of about 12 mol of ^{32}P per 48 000 g of T-substrate. The specific activity of the ^{32}P incorporated into the phosphoprotein was 860 cpm/pmol. Incubation was conducted for 10 min in the presence of 0.1 unit of each of the kinases. NMP = AMP, GMP, UMP, or CMP. WGK, wheat germ kinase; CK, casein kinase. The values have been corrected for background.

Phosphoryl Acceptor Specificity and Identification of Nucleoside Triphosphate as One of the Products of the Reverse Reaction. The ability of various nucleoside diphosphates to serve as phosphoryl acceptors for the reverse reaction was examined. The data presented in Table II show that among the various nucleoside diphosphates tested, ADP is the preferred substrate of the kinases. However, the wheat germ kinase and casein kinases II and G could also utilize GDP, IDP, and UDP as phosphoryl acceptors, as evidenced by the

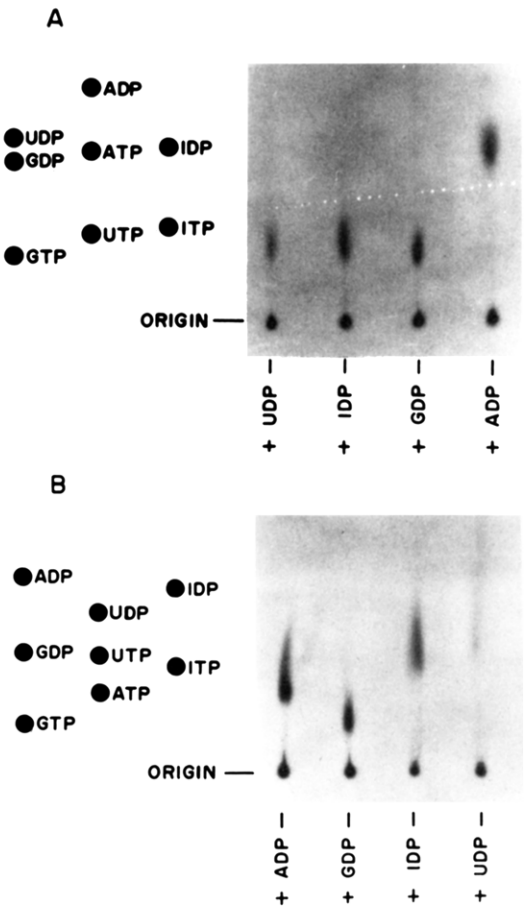

FIGURE 3: Identification of the nucleotide products of the wheat germ kinase catalyzed reverse reactions. The reverse reactions were conducted in the presence of the various nucleoside diphosphates as described in the text. The reactions were terminated by heating at 95 °C for 3 min. After centrifugation, 10 μL of the supernatants together with the markers was spotted on cellulose PEI-F thin-layer sheets (J. T. Baker Chemical Co.). The thin-layer sheets were developed by using two different solvent systems: (A) 2.0 M HCOOH/2.0 M LiCl (1:1 v/v); (B) 1.6 M LiCl. The markers were located by using an ultraviolet lamp and the radioactive spots by radioautography.

liberation of ^{32}P from ^{32}P-T-substrate in the presence of these nucleoside diphosphates. In contrast, casein kinases I and A exhibited a greater specificity with respect to the nucleoside diphosphate requirement. As shown in Table II, these kinases show only a slight activity with GDP and no activity at all with IDP and UDP. CDP and the various nucleoside monophosphates failed to elicit any release of radioactivity from ^{32}P-T-substrate.

The results presented above appear to be consistent with the properties of the kinases reported earlier on the basis of studies of the forward reaction. As shown previously, the wheat germ kinase and casein kinases II and G were found to be relatively nonspecific and could utilize either ATP or GTP to phosphorylate T-substrate (Yan & Tao, 1982b). On the other hand, casein kinases I and A were found to be specific for ATP and failed to utilize GTP as a phosphoryl donor.

In order to verify that in the kinase-catalyzed reverse reaction the ^{32}P released was incorporated into nucleoside triphosphate, the reaction product was analyzed by thin-layer chromatography in two different solvent systems. As shown in Figure 3, the reverse reaction catalyzed by the wheat germ kinase in the presence of the various nucleoside diphosphates results in the formation of the corresponding nucleoside triphosphates. The radioactive spots (as visualized by radioautography) on the thin layer were identified by comparing their

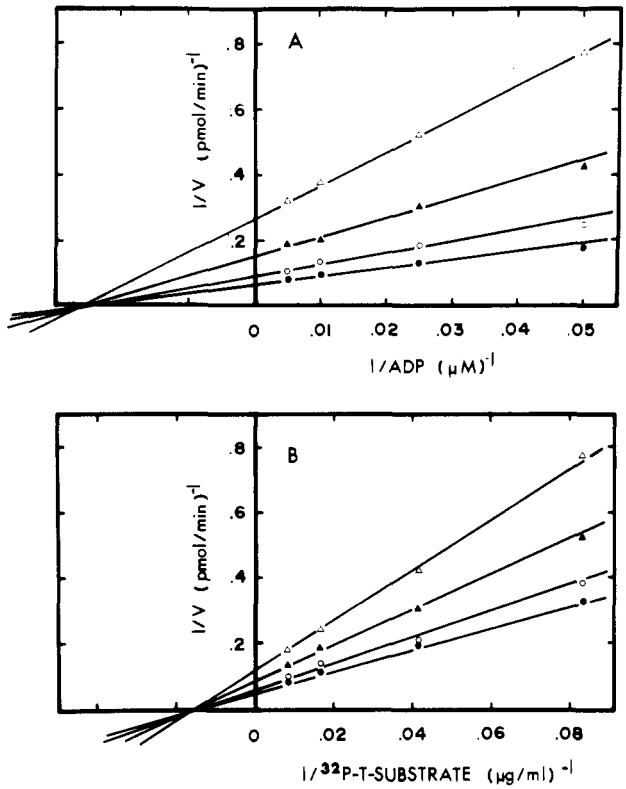

FIGURE 4: Double-reciprocal plots of the initial velocity as a function of the substrate concentration. The reverse reactions were conducted in the presence of 0.5 μg of the wheat germ kinase, and the incubation was for 5 min. (A) Double-reciprocal plots of initial velocities vs. ADP concentrations at different fixed levels of ^{32}P-T-substrate phosphorylated to the extent of about 12 mol of ^{32}P/48 000 g. The concentrations of ^{32}P-T-substrate used were 12 (△), 24 (▲), 60 (○), and 120 μg/mL (●). (B) Double-reciprocal plots of initial velocities vs. ^{32}P-T-substrate concentrations at different fixed levels of ADP. The ADP concentrations used were 0.02 (△), 0.04 (▲), 0.1 (○), and 0.2 mM (●).

mobilities with those of authentic nucleoside triphosphates added.

In another experiment, we have examined the formation of [γ-^{32}P]ATP by using a coupled enzyme reaction. The wheat germ kinase catalyzed reverse reaction with ADP and ^{32}P-T-substrate as substrates was conducted in the presence of 10 mM D-glucose and hexokinase. The coupled enzyme reaction resulted in the synthesis of glucose 6-[^{32}P]phosphate which had been identified by thin-layer chromatography (data not shown). The result indicates that the ^{32}P incorporated into the nucleotide is in the γ position which is used by the hexokinase to phosphorylate D-glucose.

We have similarly examined the nucleotide products of the reverse reaction catalyzed by other casein kinases and confirmed that they are nucleoside triphosphates.

Kinetic Studies. The kinetics of the reverse reaction catalyzed by the wheat germ kinase was examined by measuring the enzyme activity (at pH 5.8) at varying concentrations of ADP in the presence of different fixed levels of ^{32}P-T-substrate and vice versa. The double-reciprocal plots of initial velocities vs. concentrations of ADP at different fixed levels of ^{32}P-T-substrate, and vice versa, generated a series of straight lines which intersected at the left of the vertical axis and slightly below the horizontal axis (Figure 4). From the kinetic data, it appears that the transfer of phosphate from ^{32}P-T-substrate to ADP proceeds by a sequential bireactant mechanism similar to that of the forward reaction (Yan & Tao, 1982a).

The kinetic parameters of the wheat germ kinase catalyzed reverse reaction were calculated from replots of the slopes and

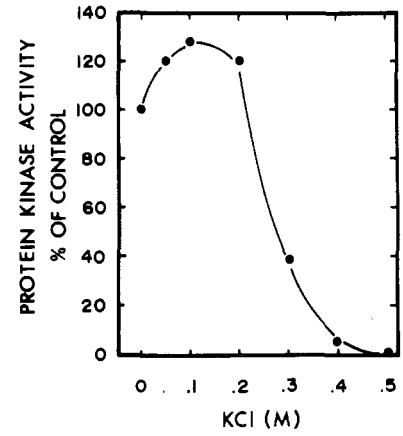

FIGURE 5: Effect of varying concentrations of KCl on the reverse reaction catalyzed by the wheat germ kinase. The reverse reaction was conducted in the presence of 0.5 μg of the kinase and 48 μg/mL ^{32}P-T-substrate (12 mol of ^{32}P/48 000 g). The incubation time was for 10 min, and the ^{32}P incorporated into the T-substrate had a specific activity of 720 cpm/pmol.

intercepts of the double-reciprocal plots in Figure 4 against the reciprocal concentrations of the nonvaried substrate (Cleland, 1970). The K_m constants for ADP and ^{32}P-T-substrate were calculated to be 22 μM and 50 μg/mL, respectively, and the V_{max} was 37 nmol of ^{32}P released min^{-1} mg^{-1}.

The equilibrium constant of the reaction catalyzed by the wheat germ kinase was determined at two different pH values. At the pH (5.8) optimum for the reverse reaction, an average value of 0.06 was obtained for K_{eq}. The K_{eq} at the pH (7.5) which favored the forward reaction was found to be about 10-fold lower, and a value of 0.008 was obtained.

Effects of Salt and Heparin on the Reverse Reaction. We have previously reported that KCl or NaCl inhibits the forward reaction catalyzed by the wheat germ kinase. About 70% of the kinase activity was inhibited at 0.2 M salt, and complete inhibition was at about 0.4 M. Although the exact mechanism by which the salt affects the kinase activity is not known, it could conceivably result from a modification of the interaction of the protein substrate with the enzyme. Since the protein substrate of the reverse reaction was highly phosphorylated and hence differed significantly from that of the forward reaction, it was of interest to determine whether the salt had a similar or a different effect on the reverse reaction. Figure 5 shows that in contrast to that observed in the forward reaction, the kinase activity in the reverse reaction is slightly stimulated at 0.1–0.2 M KCl. At high salt concentrations, however, the kinase activity was also inhibited. These data, together with those obtained earlier (Yan & Tao, 1982a), suggest that the salt may influence the kinase activity by affecting the interaction between the kinase and its substrate.

The effect of heparin on the reverse reaction was investigated. Heparin was found to inhibit strongly the reverse reaction (Figure 6). This effect is similar to that observed earlier for the forward reaction (Yan & Tao, 1982a).

Phosphoprotein Substrate Specificity. The reverse reaction was investigated by using phosphoproteins obtained from the phosphorylation of protein substrates by the same kinases used in the reverse reaction or by other kinases. The dephosphorylation of ^{32}P-casein and ^{32}P-phosvitin by the wheat germ kinase is shown in Figure 7. These phosphoproteins were maximally phosphorylated in the presence of the wheat germ enzyme. The dephosphorylation of ^{32}P-casein and ^{32}P-phosvitin appeared to be a slow process and did not reach completion. Incubation up to 12 h released only about 65% and 30% of the radioactivity from ^{32}P-casein and ^{32}P-phosvitin, respectively.

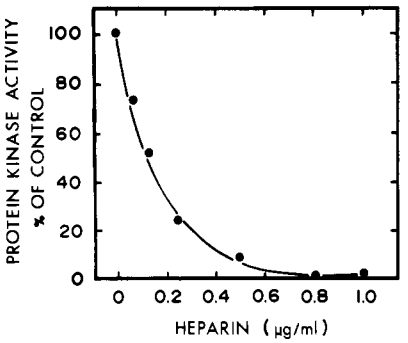

FIGURE 6: Effect of heparin on the reverse reaction catalyzed by the wheat germ kinase. The experimental conditions were as described under Figure 5.

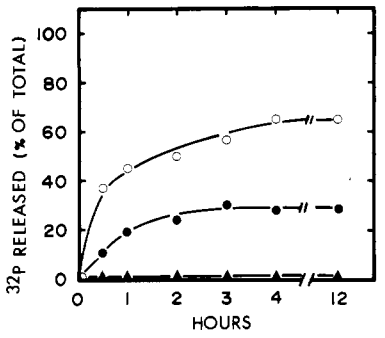

FIGURE 7: Time course of dephosphorylation of ^{32}P-casein and ^{32}P-phosvitin by the wheat germ kinase. The reaction mixture, in 0.3 mL, contained 1.2 units of the wheat germ kinase and either 0.42 mg/mL ^{32}P-casein [9.6 nmol of ^{32}P/mg (○)] or 0.6 mg/mL ^{32}P-phosvitin [23.7 nmol of ^{32}P/mg (●)]. These phosphoproteins were labeled with the wheat germ kinase. As a control (▲), a reaction mixture containing no ADP was also incubated under the same conditions in order to monitor possible nonspecific release of ^{32}P from ^{32}P-labeled phosphoproteins. Incubation was conducted at 37 °C. At each time point, an aliquot of 25 μL was withdrawn for determination of the ^{32}P released into the trichloroacetic acid soluble fraction. The total amount of radioactivity added to the reaction mixture in the form of ^{32}P-labeled phosphoprotein is taken as 100%.

The failure to reach completion was not due to the rapid inactivation of the enzyme during incubation. We found that over 80% of the enzyme activity still remained after incubation for 8 h at 37 °C. In contrast, a similar study using ^{32}P-T-substrate showed that the dephosphorylation of the phosphoprotein occurred at a significantly greater rate. Moreover, complete dephosphorylation of the substrate was observed within 2 h (Figure 8A).

The studies described above utilized the same enzyme in the phosphorylation and dephosphorylation reactions. It was therefore of interest to determine whether a kinase could also catalyze the dephosphorylation of a phosphoprotein derived from another kinase. Figure 8 shows the dephosphorylation of three different ^{32}P-T-substrate preparations by the various kinases. All these ^{32}P-T-substrate preparations were phosphorylated to saturation except with the use of different kinases. The results show that the wheat germ kinase derived ^{32}P-T-substrate is completely and rapidly dephosphorylated by the wheat germ kinase (Figure 8A). Casein kinases II and G could also dephosphorylate the ^{32}P-T-substrate but at a lower rate. Over 90% of the ^{32}P was found to be released into the trichloroacetic acid soluble fraction by these kinases. In contrast, casein kinases I and A catalyzed the release of no more than 50% of the ^{32}P from the wheat germ kinase derived ^{32}P-T-substrate. The cyclic AMP dependent protein kinase failed to elicit any activity toward the phosphoprotein.

The activity of the various kinases toward the ^{32}P-T-substrate derived from the phosphorylation by casein kinase II

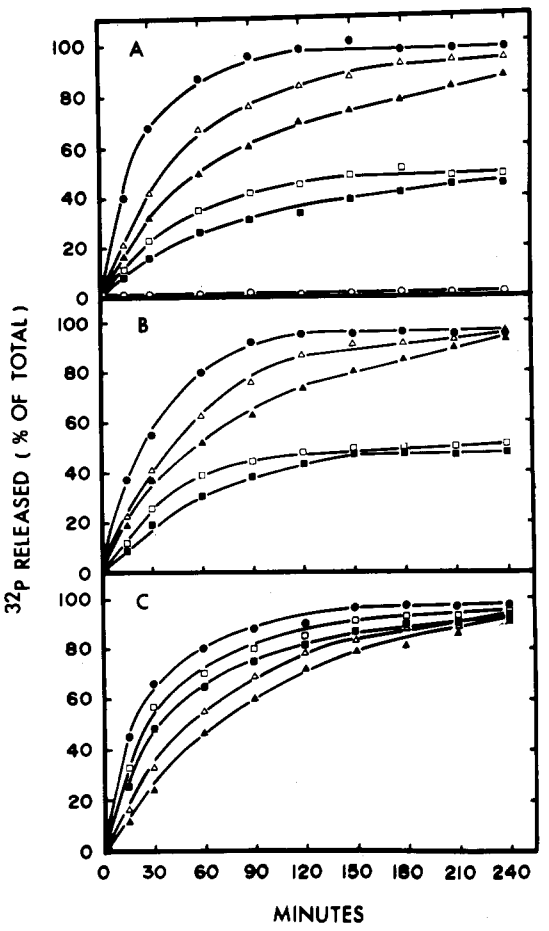

FIGURE 8: Time course of dephosphorylation of ^{32}P-T-substrates by various protein kinases. The concentrations of the phosphorylated substrates in the dephosphorylation reactions were (A) 40 μg/mL ^{32}P-T-substrate (12 mol of ^{32}P/48 000 g) derived from the action of the wheat germ kinase, (B) 39 μg/mL ^{32}P-T-substrate (12 mol of ^{32}P/48 000 g) derived from casein kinase II, and (C) 45 μg/mL ^{32}P-T-substrate (6 mol of ^{32}P/48 000 g) derived from casein kinase I. The protein kinases used were the following: 0.6 unit of wheat germ kinase (●); 1.2 units of casein kinase II (△), casein kinase I (□), casein kinase A (■), or casein kinase G (▲); and 27 μg of the catalytic subunit of the cyclic AMP dependent protein kinase (○). The ^{32}P released from the reaction mixtures containing no ADP at the various time points was found to be less than 1% of the total ^{32}P added. Other experimental details are as described under Figure 7.

Table III: Phosphoprotein Substrate Specificity of the Reverse Reactions[a]

phosphoprotein	^{32}P released (pmol/min)				
	WGK	CK-II	CK-G	CK-I	CK-A
^{32}P-T-substrate (WGK)	11.3	3.5	2.6	1.9	1.3
^{32}P-T-substrate (CK-II)	10.3	3.4	2.7	2.0	1.4
^{32}P-T-substrate (CK-I)	10.5	1.8	1.4	4.0	3.0
^{32}P-casein (WGK)	2.7	1.0	1.1	1.1	0.9
^{32}P-phosvitin (WGK)	0.8	0.4	0.3	0.3	0.2

[a] The concentration of ^{32}P-T-substrate in the reaction mixture was adjusted to contain about 12 nmol of ^{32}P/mL. The kinase used for the synthesis of the labeled phosphoprotein is indicated in parentheses. Other experimental conditions are as described under Table II.

is shown in Figure 8B. The dephosphorylation patterns, in general, resembled those of the wheat germ kinase derived ^{32}P-T-substrate.

When the ^{32}P-T-substrate derived from phosphorylation by casein kinase I was used as substrate, all the kinases were found to catalyze the complete dephosphorylation of the phospho-

protein but at varying rates (Figure 8C).

Table III compares the relative rates of dephosphorylation of ^{32}P-casein, ^{32}P-phosvitin, and ^{32}P-T-substrate by the various kinases. ^{32}P-Casein and ^{32}P-phosvitin were the products of the wheat germ kinase catalyzed reaction. On the other hand, the ^{32}P-T-substrates used were derived from the phosphorylation by three separate kinases, the wheat germ kinase, the casein kinase I, and the casein kinase II, as indicated in the table. The results show that the wheat germ kinase is significantly more active than the other kinases toward the various phosphoproteins, including those phosphoproteins which are derived from other kinases. Casein kinases I and A dephosphorylated at a higher rate the ^{32}P-T-substrate derived from casein kinase I. An examination of the table shows that there is considerable similarity in the activity of casein kinases II and G.

Discussion

Rabinowitz & Lipmann (1960) first showed that the phosphorylation of phosvitin and casein by the yeast and calf brain kinases was reversible. Thus, these enzymes were found to catalyze the transfer of phosphate from the phosphoproteins to ADP. Following this initial observation, reversibility of the kinase reaction was also demonstrated for the cyclic AMP dependent protein kinase by using the kinase's regulatory subunit (Rosen & Erlichman, 1975), casein (Shizuta et al., 1975), or pyruvate kinase (El-Maghrabi et al., 1980) as substrate. The reaction catalyzed by phosphorylase kinase was also reversible (Shizuta et al., 1977). However, the reverse reaction was found to occur only in the presence of glucose—a requirement which was not observed in the forward reaction.

In the present study, we examine the reversibility of the reactions catalyzed by five different casein kinases isolated from three different sources. These kinases can be divided into two distinct classes based on their phosphoryl donor specificities. The wheat germ kinase and casein kinases II and G can utilize both ATP and GTP as phosphoryl donors. In contrast, casein kinases I and A exhibit a strong preference for ATP. This difference in substrate specificity of the kinases is also reflected in the nucleoside diphosphate requirement in the reverse reaction.

Although the dephosphorylation of phospho-T-substrate catalyzed by the wheat germ kinase is relatively rapid and can reach completion, the physiological significance of the reverse reaction remains unknown. However, it should be pointed out that the reverse reaction exhibits a pH optimum of about 5.8 as compared to 7–8.5 for the forward reaction. This observation alone would suggest that the reverse reaction is probably physiologically insignificant. Moreover, studies have shown that the dephosphorylation of phosphoproteins is normally catalyzed by either specific or nonspecific phosphoprotein phosphatases (Lee et al., 1980).

We have previously shown that the T-substrate contains multiple phosphorylation sites (Yan & Tao, 1982b). A recent estimate indicates that the homogeneous T-substrate preparation contains approximately 2.5 mol of protein-bound phosphate (data not shown) and can be further phosphorylated to the extent of about 12 mol of phosphate per 48 000 g of T-substrate by the wheat germ kinase or by casein kinase II or G, and about 6 mol of phosphate per 48 000 g of T-substrate by casein kinase I or A. Although each of the kinases can catalyze the incorporation of several phosphate molecules into the T-substrate, it remains to be determined whether phosphorylation by the kinases occurs at distinct sites or whether common phosphorylation sites exist for the various kinases. In order to gain insight into the relationship of the various phosphorylation sites of the kinases, a detailed study of the reverse reaction using different ^{32}P-T-substrate preparations has been conducted. The rationale behind these studies is based on the notion that if two different kinases phosphorylate the same sites on the T-substrate, the phosphoprotein synthesized by one kinase via the forward reaction should be able to serve as substrate of another in the reverse reaction. Our results suggest that the sites of phosphorylation on T-substrates by the wheat germ kinase, casein kinase II, and casein kinase G are identical. These enzymes are capable of completely dephosphorylating the ^{32}P-T-substrate derived from any of the kinases. In a similar study with kinases I and A, it appears that these two enzymes recognize and phosphorylate the same phosphorylation sites on the T-substrate. However, the wheat germ kinase and casein kinases II and G can completely dephosphorylate the ^{32}P-T-substrate derived from either casein kinase II or casein kinase A, but not vice versa. Thus, among the 12 sites on the T-substrate which are phosphorylated by the wheat germ kinase, kinase II, and kinase G, 6 of these sites are also recognized by casein kinases I and A. These studies show that the relationships of the various kinases and their phosphorylation sites can be evaluated by examining the phosphoprotein substrate specificity of the reverse reaction.

Finally, it should be noted that the endogenous phosphates associated with the T-substrate were not dephosphorylated by the wheat germ kinase via the reverse reaction (data not shown). The origin of these phosphates remains unknown but could conceivably arise from the action of kinases with specificities different from those of the enzymes described in this study.

Registry No. Protein kinase, 9026-43-1; casein kinase, 52660-18-1.

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