provide some guidelines for the application of the technique to other macromolecules.

ACKNOWLEDGMENTS

We thank Drs. P. N. T. Unwin, C. W. Akey, and R. O. Fox for their helpful comments, Dr. E. P. Gogol for the use of his plotting program, and the Mass Spectrometry Resource facility (UCSF) for LSIMS spectra.

Registry No. PE, 4004-05-1; dATP, 1927-31-7; t-BOC-NH-$(CH_2)_5CO_2H$, 6404-29-1; t-BOC-aminocaproyl-PE, 110775-22-9; aminocaproyl-PE, 110796-31-1; dATP–aminocaproyl-PE, 110796-32-2.

REFERENCES

Amos, L. A., Henderson, R., & Unwin, P. N. T. (1982) *Prog. Biophys. Mol. Biol. 39*, 183–231.

Berglund, O., & Eckstein, F. (1974) *Methods Enzymol. 34B*, 253–261.

Brown, N. C., & Reichard, P. (1969) *J. Mol. Biol. 46*, 39–55.

Brown, N. C., Canellakis, Z. N., Lundin, B., Reichard, P., & Thelander, L. (1969) *Eur. J. Biochem. 9*, 561–573.

Dittmer, J. C., & Lester, R. L. (1964) *J. Lipid Res. 5*, 126–127.

Follman, H. (1982) *Naturwissenschaften 69*, 75.

Gaines, G. L., Jr. (1966) *Insoluble Monolayers at Liquid–Gas Interfaces* (Prigogine, I., Ed.) Wiley-Interscience, New York.

Joelson, T., Uhlin, U., Eklund, H., Sjoberg, B.-M., Hahne, S., & Karlsson, M. (1984) *J. Biol. Chem. 259*, 9076–9077.

Knorre, D. G., Kurbatov, V. A., & Samukov, V. V. (1976) *FEBS Lett. 70*, 105–108.

Ludwig, S. D., Ribi, H. O., Schoolnik, G. K., & Kornberg, R. D. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8585–8588.

Martin, D. W., Jr., & Gelfand, E. W. (1981) *Annu. Rev. Biochem. 50*, 845–877.

McClare, C. N. F. (1971) *Anal. Biochem. 39*, 527–530.

Möbius, D., Bücher, H., Kuhn, H., & Sondermann, J. (1969) *Ber. Bunsen-Ges. Phys. Chem. 73*, 845.

Moroder, L., Hallett, A., Wünsch, E., Keller, O., & Wersin, G. (1976) *Hoppe-Seyler's Z. Physiol. Chem. 357*, 1651–1653.

Paltauf, F. (1976) *Chem. Phys. Lipids 17*, 148–154.

Reichard, P., & Ehrenberg, A. (1983) *Science (Washington, D.C.) 221*, 514–519.

Ribi, H. O., Ludwig, D. S., Mercer, L., Schoolnik, G. K., & Kornberg, R. D. (1987) *Science (Washington, D.C.)* (submitted for publication).

Six, H., Uemura, K., & Kinsky, S. C. (1973) *Biochemistry 12*, 4003–4011.

Thelander, L., & Reichard, P. (1979) *Annu. Rev. Biochem. 48*, 133–158.

Uzgiris, E. E., & Kornberg, R. D. (1983) *Nature (London) 301*, 134–136.

Evidence for the Regulation of Protein Synthesis by a Wheat Germ Phosphoprotein Factor†

Lu Ann Aquino and Mariano Tao*

Department of Biological Chemistry, University of Illinois at Chicago, College of Medicine, Chicago, Illinois 60612

Received June 2, 1987; Revised Manuscript Received July 30, 1987

ABSTRACT: A 48-kilodalton phosphoprotein, termed T-protein or pT, isolated from wheat germ and purified to homogeneity is found to inhibit the translation of tobacco mosaic virus (TMV) RNA in both wheat germ and reticulocyte lysates. The translation of TMV RNA in both systems was inhibited over 80% by 8 μM pT. There was no evidence to indicate that the reticulocyte lysate also contained a pT-like protein. pT was rapidly phosphorylated in the wheat germ and reticulocyte lysates. Although the relationship between pT phosphorylation and inhibition of protein synthesis is not known, there is evidence to indicate that complete phosphorylation of pT is not required for inhibition. Furthermore, no significant differences in the kinetics of inhibition of protein synthesis between prephosphorylated and unmodified pT were observed. Investigation of the mechanism of inhibition indicated that neither the aminoacylation of tRNA nor the elongation of nascent polypeptide chains was affected by pT. On the other hand, pT was found to prevent the formation of the 80S initiation complex. This action of pT was not due to the binding of pT to the ribosomes. However, the effect of pT was found to vary with the concentrations and types of mRNA used in the translational system. These results suggest that pT may interact with specific region(s) of the mRNA and prevent its translation. Alternatively, pT could block the translation of mRNA by binding to one or more of the initiation factors that interact with mRNA to facilitate mRNA binding to the 43S preinitiation complex. On the basis of the action of pT, it is tempting to speculate that this protein could play an important physiological role in contributing to the dormancy of the wheat seed.

Although gene expression in both procaryotes and eucaryotes is controlled to a large extent at the level of transcription, there is increasing evidence also for control at the level of translation. In light of the complexity of the translational process, it is likely that the regulation of translation is also complex, with different mechanisms operating at different steps. Our understanding of the regulation of translation has been derived mainly from studies of the nonnucleated eucaryotic cell reticulocyte [see reviews by de Haro et al. (1985) and Moldave (1985)]. In the reticulocyte, the synthesis of the α and β chains of globin is markedly dependent on the presence

† This work was supported by Grant DK-23045 from the National Institutes of Health.

0006-2960/87/0426-7979$01.50/0

of heme. Heme deficiency has been shown to activate an inhibitor which blocks the initiation of protein synthesis. This inhibitor has been identified as a cyclic nucleotide independent protein kinase which specifically phosphorylates the α subunit of the initiation factor eucaryotic initiation factor 2 (eIF-2).[1] Another protein kinase, activated by double-stranded RNA, has also been found to phosphorylate eIF-2α. In both instances, the phosphorylation of eIF-2α prevents the recycling of the initiation factor and prevents it from acting catalytically in the initiation process. There is evidence to indicate that protein synthesis may also be regulated by the phosphorylation of the S6 protein of the 40S ribosomal subunit by a mitogen-stimulated S6 kinase (Palen & Traugh, 1987) and of aminoacyl-tRNA synthetases by casein kinase I (Pendergast & Traugh, 1985).

In contrast, there is a paucity of information concerning the regulation of protein synthesis in plant systems. Ranu (1980) has reported that a partially purified wheat germ kinase could phosphorylate the α subunit of eIF-2 from wheat germ and rabbit reticulocyte. Addition of this kinase to rabbit reticulocyte lysate resulted in the inhibition of protein synthesis which could be relieved by wheat germ eIF-2. However, the effect of the kinase on wheat germ protein synthesis was not determined. In another study, Rychlik et al. (1980) observed that the addition of a purified wheat germ kinase (M_r = 20000) to a wheat germ cell-free system inhibited the translation of Brome mosaic viral RNA. Inhibition was linked to the phosphorylation of two ribosome-associated proteins. Recently, Browning et al. (1985) showed that the addition of a purified wheat germ kinase (M_r = 38000) to a reconstituted wheat germ protein-synthesizing system could partially inhibit protein synthesis directed by yeast RNA but not by poly(uridylic acid). The kinase was found to phosphorylate eIF-2, eIF-3, and 60S ribosomal proteins. However, addition of the unphosphorylated forms of the initiation factors or 60S ribosomes failed to relieve the kinase-induced inhibition.

In our laboratory, we have examined the effect of a phosphoprotein purified to homogeneity from wheat germ lysate on protein synthesis. This phosphoprotein, termed pT or T-protein, has been previously identified as a substrate of a 38-kDa wheat germ kinase (Yan & Tao, 1982a,b). Studies have shown that pT contains as many as 10–12 phosphorylation sites and is by far the best substrate identified to date for the type II casein kinase (Yan & Tao, 1983). In this paper, data are presented which indicate that pT may play an important role in the regulation of protein synthesis.

Experimental Procedures

Materials. The wheat germ used for the preparation of the cell-free lysates was kindly supplied by General Mills, Vallejo, CA, and that for the isolation of the kinase and pT by the Dixie-Portland Co., Chicago, IL. Satellite tobacco necrosis viral RNA and tobacco mosaic virus were generous gifts from Dr. John Clark (University of Illinois, Urbana) and Dr. Milton Gordon (University of Washington, Seattle), respectively. TMV RNA was prepared from the virus by the phenol extraction method of Haschemeyer et al. (1959). VSV RNA was kindly donated by Dr. Robert Storti (University of Illinois, Medical Center). MS2 RNA, creatine kinase, and creatine phosphate were purchased from Boehringer-Mannheim Biochemicals. Phosvitin and BSA were obtained from Calbiochem-Behring. [γ-^{32}P]ATP, nuclease-treated rabbit reticulocyte lysate, and radioactive amino acids were obtained from Amersham Corp. Phosphocellulose P11 was obtained from Whatman. Sephacryl S-200 and other reagents were purchased from Sigma.

Assay for pT. pT was assayed on the basis of its ability to serve as a phosphoryl acceptor in a reaction catalyzed by the purified wheat germ kinase according to the procedure described by Yan and Tao (1982b). The reaction mixture (0.2 mL) contained 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 0.2 mM [γ-^{32}P]ATP, 0.5 unit (unless indicated otherwise) of wheat germ kinase, and pT. Incubation was carried out at 37 °C for 10 min and the reaction terminated by the addition of 2 mL of 10% trichloroacetic acid. To this quenched reaction mixture, 0.2 mg of bovine serum albumin was added as a coprecipitant, and the precipitate was collected on a Whatman GF/C glass filter. The filter was washed with 10% trichloroacetic acid, and the radioactivity on the filter was determined by counting in a liquid scintillation spectrometer.

Preparation of Wheat Germ Kinase and pT. The wheat germ kinase was purified and assayed according to the procedures of Yan and Tao (1982a). The kinase preparations used in these studies were judged to be homogeneous on the basis of SDS–polyacrylamide gel electrophoresis. One unit of kinase activity was defined as that amount of enzyme which catalyzed the incorporation of 1 nmol of phosphate into casein per minute.

The procedure for the purification of pT as described earlier by Yan and Tao (1982b) was modified as follows. The pT obtained from the phosphocellulose column was applied to a QAE-Sephadex column [2.2 × 40 cm) which had been equilibrated in a buffer [buffer A: 20 mM Tris-HCl, pH 7.5, 0.05 mM phenylmethanesulfonyl fluoride (PMSF), and 15 mM mercaptoethanol] containing 0.2 M KCl. The column was washed with 750 mL of buffer A containing 0.3 M KCl and eluted with a linear KCl gradient of 0.3–0.6 M containing 0.54 mM DFP in a total volume of 800 mL. Fractions of 5 mL each were collected and assayed for pT. The purity of each fraction was analyzed by SDS–polyacrylamide gel electrophoresis. Those fractions of pT containing only minor contaminants were pooled and applied to a Sephacryl S-200 column (2.5 × 115 cm). The column was eluted with buffer A containing 1 M KCl. Fractions of 3 mL each were collected and assayed for pT. Those fractions of pT containing only minor contaminants (as determined by SDS–polyacrylamide gel electrophoresis) were pooled, diluted to 0.1 M KCl with 20 mM Tris-HCl, pH 7.5, and applied to a second phosphocellulose column (1.2 × 18 cm) equilibrated in 20 mM Tris-HCl, pH 7.5. The column was washed with 200 mL of buffer A containing 0.2 M KCl and eluted with a linear KCl gradient of 0.2–0.6 M containing 0.54 mM DFP in a total volume of 200 mL. Fractions containing 1.5 mL each were collected and assayed for pT. The fractions were analyzed by SDS–polyacrylamide gel electrophoresis, and those exhibiting only a pT band were pooled, concentrated in an Amicon ultrafiltration cell equipped with a PM-30 membrane, and dialyzed against 20 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes)/KOH, pH 7.5, 0.2 M KCl, and 0.54 mM DFP. Protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Preparation of Phosphorylated pT. The phosphorylation of pT was conducted in a reaction mixture (5 mL) containing 50 mM Tris-HCl, pH 7.5, 0.2 mM unlabeled ATP or [γ-

[1] Abbreviations: PMSF, phenylmethanesulfonyl fluoride; DFP, diisopropyl fluorophosphate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; CTAB, cetyltrimethylammonium bromide; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; TMV, tobacco mosaic virus; eIF, eucaryotic initiation factor; kDa, kilodalton(s); BSA, bovine serum albumin.

Table I: Purification of T-Protein from Wheat Germ

step	volume (mL)	total protein (mg)	total act.[a] (nmol)	sp act. (nmol/mg)	x-fold purification	yield (%)
crude extract[b]	8000	608000	208000	0.34	1	100
DEAE-cellulose	650	13748	61750	4.50	13.2	29.6
phosphocellulose	225	674	17599	26.0	76	8.5
QAE-Sephadex	118	292	8968	30.7	90.3	4.5
Sephacryl S-200	30	44.7	1542	34.5	101	0.74
second phosphocellulose	20	11.6	714	61.6	181.2	0.34

[a] T-protein activity was defined as nanomoles of ^{32}P incorporated into trichloroacetic acid insoluble materials under the assay conditions described in the text. The phosphorylation of T-protein was conducted to saturation. The proportion of phosphate incorporated into pT represents about 16% of the total incorporated into proteins. [b] Crude extract was prepared from 3 kg of sieved wheat germ.

^{32}P]ATP (4000 cpm/pmol), 5 mM $MgCl_2$, leupeptin (2 mg), 0.67 mM DFP, and wheat germ kinase (2 units/mL). The reaction mixture was incubated at 37 °C for 30 min. The unreacted ATP was removed by gel filtration on a Sephacryl S-200 column (1.5 × 95 cm). The column was eluted with buffer A containing 1 M KCl. The procedure resulted in the incorporation of 9–10 mol of phosphate/mol of pT.

Wheat Germ Cell-Free Protein Synthesis. The wheat germ extract was prepared according to the method of Anderson et al. (1983). The assay for polypeptide polymerization was conducted as described by Pelham and Jackson (1976) with slight modifications. The reaction mixtures in 25 μL contained 7.5 μL of wheat germ extract (A_{260nm} = 100), 1.2 mM ATP, 0.1 mM GTP, 5.5 mM creatine phosphate, 5 μg of creatine kinase, 0.3 mM dithiothreitol, 28 mM Hepes/KOH, pH 7.5, 80 μM spermidine phosphate, 50 μM each of 19 unlabeled amino acids, 0.17 mM DFP, 60 pmol of [^{35}S]methionine [(1.4–3.3) × 10^5 cpm/pmol], and 1.5 μg of TMV RNA. The final concentrations of K^+ and Mg^{2+} in the reaction mixtures were 146 and 2.2 mM, respectively. Incubations were carried out at 25 °C. One-microliter samples were withdrawn before the incubations were started (0 time control) and thereafter at specified time intervals. Hot trichloroacetic acid precipitable radioactivity was measured according to the method of Voorma et al. (1979).

Rabbit Reticulocyte Cell-Free Protein Synthesis. The assay for polypeptide polymerization directed by TMV RNA was conducted according to the instructions provided with the nuclease-treated rabbit reticulocyte lysate purchased from the Amersham Corp. The reaction mixtures (25 μL) contained 7.5 μL of rabbit reticulocyte lysate, 60 pmol of [^{35}S]methionine [(1.4–3.3) × 10^5 cpm/pmol], and 1.5 μg of TMV RNA. The final concentrations of potassium and magnesium were 108 and 2 mM, respectively. Incubations were carried out at 30 °C. Samples were withdrawn and processed for radioactivity according to the method described in the preceding section. However, after the final ether wash, 10% H_2O_2 (50 μL) was added to each disk to decolorize the samples. The disks were dried under an infrared lamp and counted in 5 mL of toluene-based scintillation fluid.

Poly(uridylate)-Directed Poly(phenylalanine) Biosynthesis. Poly(uridylate) [poly(U)]-directed polymerization of phenylalanine was conducted as described above with slight modifications. The reaction mixtures (25 μL) contained 7.5 μL of cell-free extract (A_{260nm} = 100), 23 pmol of [^3H]phenylalanine (1.7 × 10^5 cpm/pmol), 0.3 mM dithiothreitol, 28 mM Hepes/KOH, pH 7.5, 1.2 mM ATP, 0.1 mM GTP, 5.5 mM creatine phosphate, 5 μg of creatine kinase, 0.17 mM DFP, and 10 μg of poly(U). The final concentrations of K^+ and Mg^{2+} were 146 and 8 mM, respectively.

Sucrose Density Gradient Centrifugation. The analysis of the protein-synthesizing reaction mixtures by sucrose density gradient centrifugation was conducted on 5 mL of a linear (15–30%) sucrose density gradient prepared in a buffer containing the following: 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 3 mM $Mg(OAc)_2$, and 0.3 mM dithiothreitol. The reaction mixtures (0.1 mL) were diluted with 0.15 mL of cold gradient buffer before being layered on the gradients. Centrifugation was conducted at 2 °C in a Beckman SW 50.1 rotor at 45 000 rpm for 2 h. Fractions of 0.15 mL were collected from the bottom of the tube. Aliquots of 0.1 mL were removed from each fraction and diluted with 0.3 mL of deionized H_2O. The absorbance of the diluted samples was monitored at 260 nm and processed for cetyltrimethylammonium bromide (CTAB)-precipitable radioactivity according to the method of Darnbrough et al. (1973).

SDS–Polyacrylamide Gel Electrophoresis. SDS–polyacrylamide gel electrophoresis was conducted according to the method of Laemmli (1970). The molecular weight markers used were phosphorylase *b* (94 000), bovine serum albumin (68 000), ovalbumin (43 000), carbonic anhydrase (29 000), and cytochrome *c* (17 200).

Results

Purification of pT. The overall purification procedure of pT is summarized in Table I. From 3 kg of starting material, 11.6 mg of purified pT was obtained which represented a yield of about 0.34%. The low recovery may be due to two factors. One is the presence of other phosphoryl acceptor activities in the crude extract, and the other is the result of our strategy to sacrifice yield for purity by pooling only those fractions obtained from several column chromatographic steps which exhibit the least contaminants. The purity of the final pT preparation was assessed by SDS–polyacrylamide gel electrophoresis and found to contain only a single Coomassie blue staining band. This protein band was phosphorylated when incubated in the presence of the wheat germ kinase and [γ-^{32}P]ATP. Control experiments in which the kinase or pT alone was incubated with [γ-^{32}P]ATP failed to reveal any phosphorylated bands. The result indicates that the radiolabeled protein band seen in the complete phosphorylation reaction mixture is not derived from the kinase preparation or from the autophosphorylation of pT due either to an inherent kinase activity associated with pT or to contaminating kinase activity in the pT preparation.

Inhibition of Wheat Germ Protein Synthesis by pT. During the course of our investigation of the role of the kinase in the regulation of cellular processes in wheat germ, we found that several components of the translational system were phosphorylated by the enyme (Browning et al., 1985). However, the significance of the phosphorylation of the translational components in protein synthesis remains unknown. Since pT could also serve as a substrate of the kinase and appeared to be an efficient one, it was of interest to determine whether pT might play a role in the regulation of protein synthesis in wheat germ. Figure 1 shows the time course of the extent of TMV RNA directed incorporation of radiolabeled methionine into protein in the presence and absence of added pT. The control

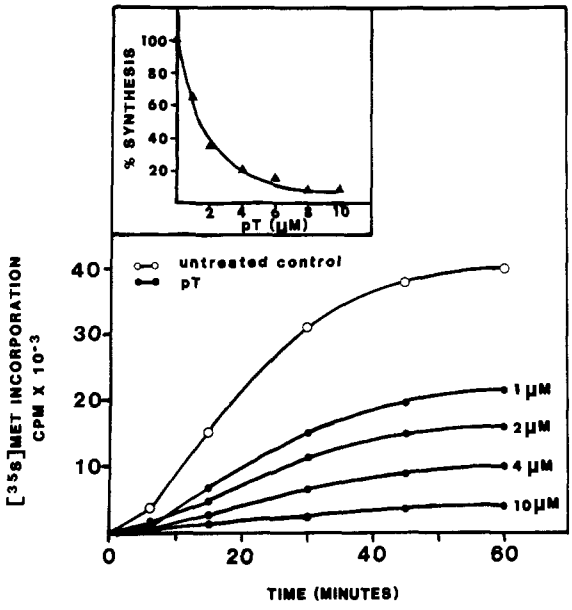

FIGURE 1: Inhibition of translation of TMV RNA in wheat germ lysate by pT. Protein synthesis was carried out in the absence (○) and presence (●) of varying concentrations of pT as described under Experimental Procedures. The percent synthesis (inset) in the samples containing pT was calculated for the 45-min incubation time point by dividing the amount of [^{35}S]methionine incorporated in the presence of pT by that in the uninhibited control sample.

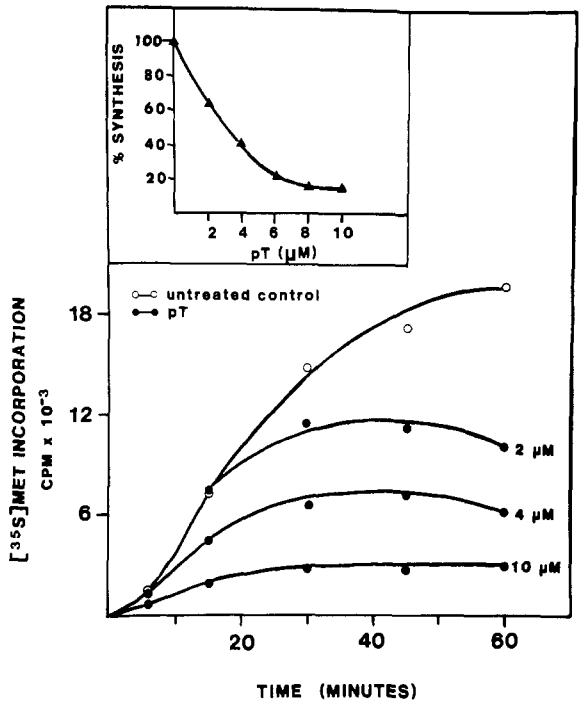

FIGURE 2: Inhibition of translation of TMV RNA in rabbit reticulocyte lysate by pT. See legend of Figure 1.

curve represents the amount of incorporation observed without addded pT. The addition of 1–10 μM pT to the lysate resulted in a concentration-dependent inhibition of protein synthesis as shown by the reduced incorporation as compared to the untreated control. As indicated by the inset figure, protein synthesis was inhibited by 50% at about 1.7 μM pT and over 90% at 8–10 μM pT. It should be noted that control experiments using bovine serum albumin and phosvitin showed that these proteins did not elicit the same inhibitory effect as pT on the translation of TMV RNA. Like pT, phosvitin is also a substrate of the wheat germ kinase and is a highly phosphorylated protein. These results suggest that the inhibition of protein synthesis by pT is specific and is not due to any nonspecific interaction of exogenous proteins with components of the translational system.

The above studies were extended to the reticulocyte lysate system in order to determine whether the effect of pT on protein synthesis was unique to the wheat germ lysate system. Figure 2 shows that pT also inhibited the incorporation of [^{35}S]methionine into TMV RNA proteins in the reticulocyte lysates. However, the data show that a somewhat higher concentration of pT was needed to achieve the same extent of inhibition as observed in the wheat germ system.

In an attempt to gain insight into the mode of action of pT, it was of interest to determine whether the inhibition of wheat germ protein synthesis was affected by preincubation of the lysate with pT. It could be argued that if preincubation of the lysate with pT led to a progressive loss of protein synthesizing activity that the inhibitory effect exerted by pT might be catalytic in nature. Our results indicate that the inhibition of protein synthesis by pT was rapid and was not enhanced by preincubation of the wheat germ lysate with pT. The percent inhibition by pT relative to the uninhibited control remained constant with respect to the time of preincubation. The preincubation of the lysate with pT was conducted in the presence of all components of the protein synthesizing system except the amino acids. Thus, the data suggest that the action of pT was probably not catalytic but might involve the direct binding to one or more components of the protein synthesizing system. It should also be noted that we have not been able to detect any ribonuclease activity associated with the pT preparation (data not shown).

Phosphorylation of pT by Endogenous Wheat Germ Lysate Kinase(s). Since pT is an excellent substrate of the purified wheat germ kinase and can be phosphorylated to the extent of about 10–12 mol of phosphate per mole of pT (Yan & Tao, 1983), it is of interest to determine whether pT is also phosphorylated by lysate kinase(s) during protein synthesis. Our experiments indicate that the added pT (10 μM) is rapidly phosphorylated in the lysate in the presence of [γ-^{32}P]ATP under conditions of protein synthesis. It should be noted that the wheat germ lysate contained endogenous pT which was also phosphorylated in the presence of [γ-^{32}P]ATP. Preliminary estimates by densitometric tracing of an SDS gel of the lysate indicated that pT represented 2–4% of the total lysate proteins. The time course of phosphorylation of pT under conditions of protein synthesis was measured by excising the protein band after SDS gel electrophoresis of the phosphorylated lysate and counting the radioactivity in the excised band. Figure 3 indicates that the phosphorylation of pT in the lysate is relatively rapid and reaches a maximal level at about 40 min of incubation. The amount of phosphate incorporated was in addition to the 2–3 mol of phosphate intrinsic to the isolated pT (Yan & Tao, 1983). A comparison of the extent of phosphorylation of pT (10 μM) with the extent of inhibition of protein synthesis suggests that maximal phosphorylation of pT is probably not required for the inhibition of protein synthesis. As shown in the figure, the additional phosphorylation of pT does not appear to have an effect on the inhibition of protein synthesis.

Incubation of rabbit reticulocyte lysate with [γ-^{32}P]ATP and 10 μM pT under conditions of protein synthesis also resulted in the phosphorylation of pT as shown in the radioautogram in Figure 4B, lane 3. This is to be expected since we have shown earlier that human erythrocytes contain an enzyme, termed casein kinase G, which can also catalyze the phosphorylation of pT at sites similar to those recognized by

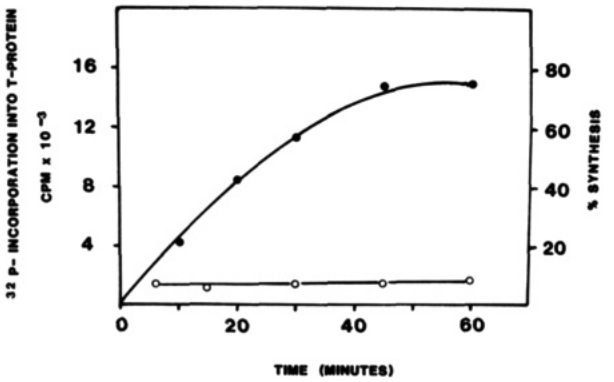

FIGURE 3: Time course of phosphorylation of pT in the wheat germ lysate. pT (10 μM) was incubated in the complete protein synthesis system in the presence of [γ-^{32}P]ATP (151 cpm/pmol) for varying time intervals. The incorporation of radiolabeled phosphate into pT was analyzed by SDS gel electrophoresis and radioautography. The amount of [^{32}P]P_i incorporated into 10 μM pT (●) was determined by excising the protein bands from the dried gel, solubilizing in 0.5 mL of tissue solubilizer (RPI Corp.), and counting in a liquid scintillation counter. The percent synthesis in the samples in parallel experiments (○) was measured and calculated as described under Figure 1.

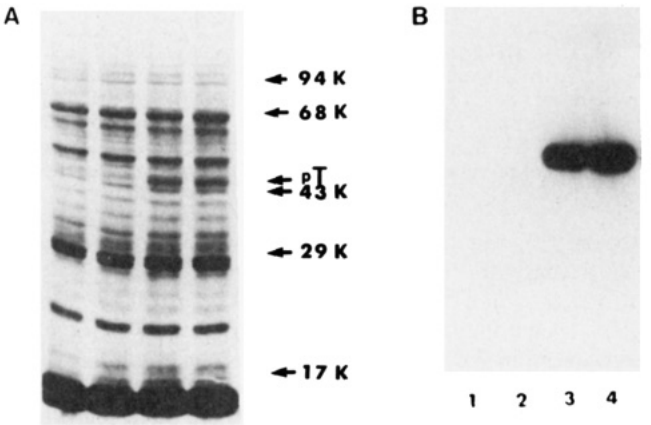

FIGURE 4: Phosphorylation of pT by the rabbit reticulocyte lysate. The complete reticulocyte lysate protein synthesizing reaction mixture (25 μL) containing [γ-^{32}P]ATP (82 cpm/pmol) (lane 1) was incubated in the absence (lane 1) or presence of wheat germ kinase (4.1×10^{-3} unit) (lane 2) or in the presence of 10 μM pT (lane 3) or 10 μM pT plus wheat germ kinase (4.1×10^{-3} unit) (lane 4) for 20 min at 30 °C. Following the incubation, a 10-μL aliquot was withdrawn, denatured by heating with SDS, and electrophoresed in a 10% polyacrylamide gel slab. The Coomassie blue stained gel profile is shown in (A). The corresponding radioautogram (B) was prepared by exposing the dried gel to X-ray film for 48 h with the aid of a Du Pont Lightning Plus intensifying screen.

the wheat germ kinase (Yan & Tao, 1983). Incubation of the lysate with [γ-^{32}P]ATP in the absence (lane 1) and presence (lane 2) of wheat germ kinase indicates that the reticulocyte lysate does not appear to contain a pT-like protein. Lane 4 shows that the addition of wheat germ kinase to lysate containing [γ-^{32}P]ATP and 10 μM pT slightly enhances the phosphorylation of pT. The corresponding Coomassie blue stained gel profiles of the reticulocyte lysate proteins with and without wheat germ kinase in the absence and presence of 10 μM pT are presented in Figure 4A.

In order to further assess the significance of the phosphorylation of pT on its ability to regulate protein synthesis, experiments were performed in which the effect of prephosphorylated pT on the translation of TMV RNA was examined. pT was prephosphorylated to the extent of about 10 mol of phosphate per mole of pT with the purified wheat germ kinase. The amount of phosphate incorporated was in addition

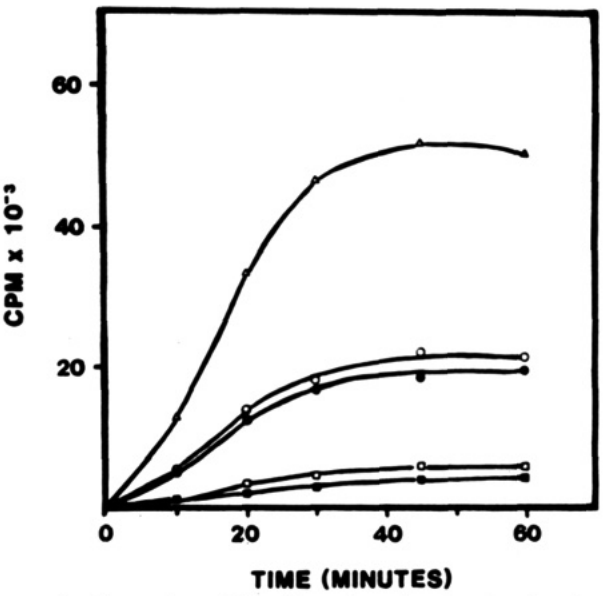

FIGURE 5: Comparison of the effect of prephosphorylated and unmodified pT on the translation of TMV RNA. Translation of TMV RNA was carried out in the absence (△) and presence of unmodified [(○) 2 μM; (□) 8 μM] or prephosphorylated [(●) 2 μM; (■) 8 μM] pT as described under Experimental Procedures. The prephosphorylated pT contained 9–10 mol of phosphate/mol of pT and was prepared by phosphorylation with the wheat germ kinase as described under Experimental Procedures.

to the 2–3 mol of phosphate that was present in the isolated protein. The results presented in Figure 5 shows that there are no significant differences in the kinetics of the inhibition of protein synthesis between prephosphorylated and unmodified pT.

Analysis of the Site of Action of pT. In order to explore the mechanism by which pT inhibited protein synthesis, the effects of pT on several of the protein synthetic steps were examined. Our first approach was to investigate the effect of pT on the aminoacylation of tRNA. The same concentrations of pT that were observed to inhibit protein synthesis were found not to affect the aminoacylation of tRNA with radiolabeled methionine. Likewise, pT was found not to inhibit the formation of other aminoacyl-tRNAs, such as Phe-, Leu-, and Thr-tRNA, or in the presence of a mixture of ^{14}C-labeled amino acids (data not shown).

The possibility that pT might be inhibiting protein synthesis by blocking polypeptide chain elongation was examined in a poly(uridylic acid) [poly(U)]-directed phenylalanine polymerization system. Studies have shown that the chain elongation step can be examined by using a poly(U)-directed poly(phenylalanine) synthesis system in the presence of high Mg^{2+} concentrations. Under these conditions, the initiation step is bypassed (Crystal et al., 1974; Spremulli et al., 1979; Ranu, 1983). The result of this study clearly indicates that pT does not inhibit poly(U)-directed poly(phenylalanine) biosynthesis, suggesting that polypeptide chain elongation is not affected by pT.

Lastly, the effect of pT on the formation of wheat germ 80S initiation complexes was examined by using sucrose density gradient centrifugation (Darnbrough et al., 1973). The formation of 80S initiation complexes, as identified by the bound [^{35}S]Met-tRNA and ^{35}S-labeled peptidyl-tRNA, was precipitated with the detergent CTAB. Figure 6b shows that incubation of wheat germ lysate with TMV RNA, [^{35}S]-methionine, and 10 μM pT for 10 min results in a markedly reduced level (by about 85%) of 80S initiation complexes formed as compared to the control with no added pT (Figure

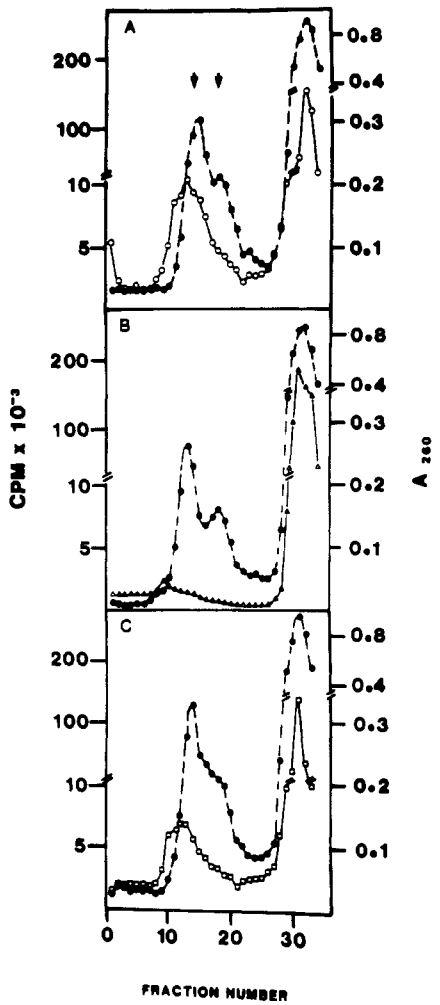

FIGURE 6: Effect of pT on 80S initiation complex formation in the wheat germ lysate system. The protein synthesizing reaction mixtures (0.1 mL) containing [^{35}S]methionine and TMV RNA were incubated for 10 min at 25 °C in the absence (A) or presence of 10 μM pT (B). Panel C represents an experiment in which the translational mixture was incubated at 25 °C for 2 min followed by the addition of 10 μM pT and further incubation for 8 min. (●) A_{260nm}; (○, △, □) radioactivity. The arrows indicate the sedimentation positions of purified 60S and 40S ribosomal subunits. The direction of sedimentation is from right to left. Other experimental details are described under Experimental Procedures.

6A). On the other hand, preincubation of the lysate with TMV RNA and [^{35}S]methionine for 2 min followed by the addition of pT and further incubation for 8 min resulted in only a 30% inhibition of complex formation (Figure 6C). These experiments suggest that pT was interfering with the assembly of the 80S initiation complex. However, pT has little or no effect on the 80S initiation complex once formed.

In a separate study, sucrose gradient analysis of lysates incubated with ^{32}P-labeled pT for 10 min indicated that there ws no observable binding of the protein to ribosomal subunits (data not shown).

Effect of TMV RNA Concentrations on the Inhibition of Polymerization by pT. Since pT did not appear to bind directly to ribosomes, it was of interest to determine whether pT could affect the formation of the 80S initiation complex by blocking interactions involving mRNA. To explore this possibility, experiments were conducted in which the concentration of TMV RNA was varied in a partially inhibited (containing 2 μM pT) and uninhibited (control) system. As shown in Figure 7, high concentrations of mRNA could overcome the inhibition by pT. The inset figure is a replot of

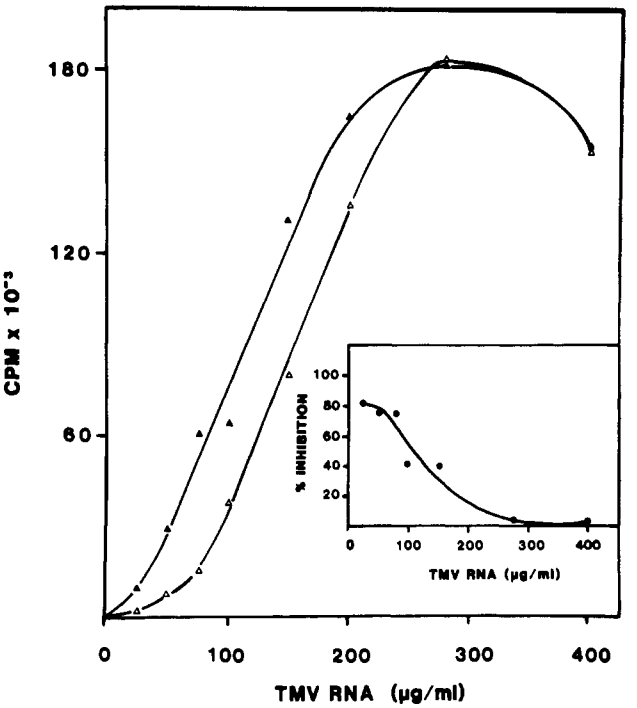

FIGURE 7: Effect of TMV RNA concentrations on the inhibition of wheat germ protein synthesis by pT. The translation of varying concentrations of TMV RNA was carried out in the absence (▲) and presence (△) of 2 μM pT. The incubation was conducted at 25 °C for 20 min. The inset figure is a replot of the data showing the percent inhibition of protein synthesis by 2 μM pT versus the concentration of TMV RNA.

the data showing the percent inhibition as a function of TMV RNA concentration.

In light of the above findings, the possibility that wheat germ tRNA could similarly relieve the inhibition caused by pT was also examined. Assuming that the average molecular mass of the different tRNA species is 25 kDa and the molecular mass of TMV RNA is 2 × 10^3 kDa, we have found that tRNA, at concentrations 40–200-fold over the concentration of TMV RNA, fails to relieve the inhibition caused by 2 μM pT.

Effect of pT on the Translation of Other Natural mRNAs. In order to determine whether inhibition of translation by pT was unique to TMV RNA, we have also examined the effects of pT on the translation of VSV RNA, MS2 RNA, and globin mRNA. The results presented in Figure 8 show that the translations of the various RNAs are inhibited to varying degrees by pT. Similar experiments showed that pT also strongly inhibited the translation of STNV RNA (data not shown). TMV RNA, which was translated most efficiently by the wheat germ lysate, was most sensitive to inhibition by pT. This was followed by VSV RNA and globin mRNA in that order. On the other hand, pT (except at high concentrations) was relatively ineffective in blocking the translation of MS2 RNA. These findings indicate that the effect of pT may vary with the type of mRNA. However, whether this selectivity is due to specific structural features of the mRNA remains to be determined.

Discussion

pT, a 48-kDa phosphoprotein, was initially identified as an endogenous substrate of the type II wheat germ casein kinase (Yan & Tao, 1982b). This protein is unique in that it contains a large number of phosphorylation sites and is an excellent substrate of the wheat germ kinase. Although the exact physiological function of pT is presently not known, the results

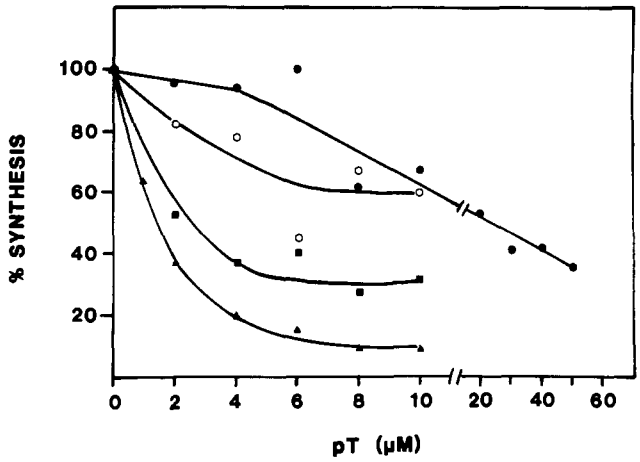

FIGURE 8: Effect of pT on the translation of various mRNAs in the wheat germ lysate. Incubations were carried out as described under Experimental Procedures in the presence of 3 μg of MS2 RNA (●), 0.52 μg of globin mRNA (○), 1.5 μg of VSV RNA (■), or 1.5 μg of TMV RNA (▲). The cpm of [^{35}S]methionine incorporated in the control samples containing no pT are as follows: MS2 RNA, 4256 cpm; globin mRNA, 2052 cpm; VSV RNA, 11 012 cpm; and TMV RNA, 41 786 cpm. The percent synthesis was calculated as described in the legend of Figure 1.

presented in this report suggest that this protein may play a role in the regulation of protein synthesis. pT was found to inhibit strongly the translation of TMV RNA in both wheat germ and reticulocyte lysates. However, the reticulocyte system was less sensitive to inhibition by pT than the wheat germ system. The reason for this difference in sensitivity between the two systems is not clear. It should be noted that there is no evidence to indicate a pT-like protein is also present in reticulocyte lysates.

No ribonuclease-associated activity had been detected in the pT preparation. It was also unlikely that the inhibition caused by pT was due to protease activity as we had included a protease inhibitor in our translational assay, and autolysis of pT was not detected. In any event, our study suggests that the mode of action of pT is not catalytic in nature. Although the exact mechanism by which pT inhibits translation is not known, we have ruled out several possible sites of action of pT. Our data clearly indicate that pT does not affect the charging of tRNA or the elongation of polypeptide chains. pT also does not appear to interact directly with ribosomes nor does it affect the functions of tRNA. On the other hand, pT was found to block the formation of the 80S initiation complex. Our data indicate, however, that pT has little or no effect on the preformed initiation complex. Obviously, an important question that remains to be resolved is the mechanism by which pT interferes with the formation of the initiation complex. A possible clue to the action of pT was the finding that inhibition by pT was not observed at high concentrations of mRNA. In addition, the effects of pT were found to vary with the type of mRNA used in the translational system. These results suggest that pT may bind to a specific region of the mRNA and prevent the translation of the mRNA. Alternatively, pT could bind to one or more of the initiation factors that interact with mRNA (Merrick et al., 1986) to facilitate mRNA binding to the 43S preinitiation complex. Increases in the concentration of mRNA could shift the equilibrium toward the formation of more mRNA–initiation factor complexes and fewer pT–initiation factor complexes, thereby increasing the rate of translation. The possibilities that pT may directly interact with mRNA or form complexes with other translational factors through protein–protein interactions are presently being investigated in our laboratory. In this regard, we have preliminary evidence which indicates that pT may bind to STNV RNA. However, a more detailed study needs to be carried out in order to determine the nature of this interaction.

Although pT was rapidly phosphorylated in the wheat germ extracts, the role of phosphorylation on its ability to inhibit the translational system is not clear. Nonetheless, complete phosphorylation does not appear to be required for the inhibitory effect of pT. Furthermore, no significant differences in the kinetics of inhibition were observed between prephosphorylated and unphosphorylated pT. It should be noted, however, that the wheat germ lysate contains phosphatase activities which are capable of dephosphorylating phospho-pT. This has been demonstrated in experiments by incubating ^{32}P-labeled pT (maximally phosphorylated) with the wheat germ lysate (data not shown). The turnover of the ^{32}P label was relatively slow, and only about half of the label was released after 1 h of incubation. Since the translational system contains endogenous protein kinase activity as well as the necessary phosphoryl donor, it is likely that pT will be rephosphorylated under the condition. On the other hand, the presence of this endogenous kinase activity in the wheat germ lysate has made it difficult to assess the significance of phosphorylation of pT. We are presently attempting to raise antibodies against the wheat germ kinase. Hopefully, the antibodies will inhibit the kinase activity in the lysate and provide a useful approach to analyze the effect of phosphorylation–dephosphorylation on the inhibitory activity of pT.

On the basis of the action of pT, it is tempting to speculate that this protein could play an important physiological role in contributing to the dormancy of wheat seed. This is an attractive hypothesis since there is evidence to indicate that mRNA is preserved in the dry embryo of wheat seeds in the form of messenger ribonucleoproteins (mRNPs) (Ajtkhozhin et al., 1976). It has also been demonstrated that protein synthesis during early germination is dependent upon this stored mRNA (Spiegel & Marcus, 1975). In addition, a free pool of RNA binding proteins appears to exist in cytoplasmic extracts prepared from dry wheat embryos which have been observed to promote the formation of mRNPs in vitro using exogenous RNA (Ajtkhozhin & Kim, 1975). Taken together, it is plausible that pT is an mRNA binding protein involved in the repression of translation of stored mRNA in the wheat embryo. Imbibition of water by the wheat seed, which activates the germination process under appropriate conditions, may also activate a protease that hydrolyzes pT (which has been found to highly susceptible to proteolysis during its isolation; unpublished data). This overall process would result in the release or "unmasking" of the stored mRNA from its nontranslatable state and its subsequent mobilization into polysomes for translation. In line with this notion, we have preliminary evidence which suggests that pT may be absent in germinating wheat seeds.

REFERENCES

Ajtkhozhin, M. A., & Kim, T. N. (1975) *FEBS Lett. 53*, 102–104.

Ajtkhozhin, M. A., Doschanov, Kh. I., & Akhanov, A. V. (1976) *FEBS Lett. 66*, 124–126.

Anderson, C. W., Straus, J. W., & Dudock, B. S. (1983) *Methods Enzymol. 101*, 635–644.

Browning, K. S., Yan, T.-F. J., Lauer, S. J., Aquino, L., Tao, M., & Ravel, J. M. (1985) *Plant Physiol. 77*, 370–373.

Crystal, R. G., Elson, N. A., & Anderson, W. F. (1974) *Methods Enzymol. 30*, 101–127.

Darnbrough, C., Legon, S., Hunt, T., & Jackson, R. J. (1973) *J. Mol. Biol. 76*, 379–403.

de Haro, C., de Herreros, A., & Ochoa, S. (1985) *Curr. Top. Cell. Regul. 27*, 63-81.
Haschemeyer, R., Singer, B., & Fraenkel-Conrat, H. (1959) *Proc. Natl. Acad. Sci. U.S.A. 45*, 313-319.
Laemmli, U. K. (1970) *Nature (London) 227*, 680-685.
Lowry, O. H., Rosenbrough, N. L., Farr, A. R., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265-275.
Merrick, W. C., Abramson, R. D., Caliendo, A. M., Grifo, J. A., & Dever, T. E. (1986) in *Translational Control* (Mathews, M. B., Ed.) pp 19-24, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Moldave, K. (1985) *Annu. Rev. Biochem. 54*, 1109-1149.
Palen, E., & Traugh, J. A. (1987) *J. Biol. Chem. 262*, 3518-3523.
Pelham, H. R. B., & Jackson, R. J. (1976) *Eur. J. Biochem. 67*, 247-256.
Pendergast, A. M., & Traugh, J. A. (1985) *J. Biol. Chem. 260*, 11769-11774.
Ranu, R. S. (1980) *Biochem. Biophys. Res. Commun. 97*, 1124-1132.
Ranu, R. S. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 3148-3152.
Rychlik, W., Kupidlowska, E., Nowak, E., & Zagorski, W. (1980) *Biochemistry 19*, 5249-5255.
Spiegel, S., & Marcus, A. (1975) *Nature (London) 256*, 228-230.
Spremulli, L. L., Walthall, B. J., Lax, S. R., & Ravel, J. M. (1979) *J. Biol. Chem. 254*, 143-148.
Voorma, H. O., Thomas, A., Goumans, H., Amesz, H., & Van Der Mast, C. (1979) *Methods Enzymol. 6*, 124-135.
Yan, T.-F. J., & Tao, M. (1982a) *J. Biol. Chem. 257*, 7037-7043.
Yan, T.-F. J., & Tao, M. (1982b) *J. Biol. Chem. 257*, 7044-7049.
Yan, T.-F. J., & Tao, M. (1983) *Biochemistry 22*, 5340-5346.

Divalent Cation and Hydrogen Ion Effects on the Structure and Surface Activity of Pulmonary Surfactant†

Hava Efrati,‡ Samuel Hawgood,*,‡,§ Mary C. Williams,‡,∥ Keelung Hong,⊥ and Bradley J. Benson#
Cardiovascular Research Institute, Departments of Pediatrics and Anatomy, and Cancer Research Institute, University of California, San Francisco, California 94143, and California Biotechnology, Inc., Mountain View, California 94043
Received August 13, 1986; Revised Manuscript Received June 8, 1987

ABSTRACT: The structure and surface activity of the extracellular fraction of pulmonary surfactant known as tubular myelin are Ca^{2+} dependent. Previous studies have demonstrated surfactant-specific proteins with monomeric molecular weights of 28 000–36 000 (SP28–36) are associated with this fraction. In reassembled lipoprotein mixtures, SP28–36 promotes the Ca^{2+}-induced aggregation and surface activity of surfactant lipids, but the detailed interactions between Ca^{2+}, SP28–36, and surfactant lipids have not been established. In this study, we investigated the effect of various cations on the aggregation of surfactant lipid liposomes in the presence of SP28–36. SP28–36 reduced the threshold ion concentration for liposome aggregation from >10 to 0.5 mM for Ca^{2+}, Ba^{2+}, and Sr^{2+} but not Mg^{2+} or Mn^{2+}. The liposome aggregation was reversed by ethylenediaminetetraacetic acid and not associated with leakage of carboxyfluorescein. SP28–36 promoted similar liposome aggregation at pH <5 in the absence of divalent cations. Surfactant lipids adsorbed slowly to an air–fluid interface in all ionic conditions unless SP28–36 was present. Both Ca^{2+} and H^+ induced rapid lipid adsorption in the presence of SP28–36. The surface activity of native surfactant had a similar ion dependence. Electron micrographs of native surfactant showed typical tubular myelin structures at pH 7.4 only in the presence of Ca^{2+}. At pH 4.4 in the absence of Ca^{2+}, similar but not identical structures were seen. In the reconstituted system, SP28–36 in the presence of Ca^{2+} induced the formation of larger multilayered structures including parallel bilayers and small areas of squares and triangles with dimensions similar to structures found in the native material. The pH at which the protein-induced changes in lipid aggregation and surface activity occur is similar to the p*I* of SP28–36. Because the marked effects of H^+ and Ca^{2+} on surfactant lipid dispersion and surface activity are similar and are only seen in the presence of SP28–36, we propose that the action of Ca^{2+} at physiological pH is related, at least in part, to neutralization of the negatively charged carboxyl groups on the protein.

Dipalmitoylphosphatidylcholine (DPPC)[1] is widely accepted to be the major component of pulmonary surface-active material (surfactant) that is responsible for the maintenance of low surface tension (Brown, 1964; Notter & Morrow, 1975; Hildebran et al., 1979). However, pure DPPC below its phase transition temperature of 41.5 °C is very slow to adsorb to and spread at an air–water interface (Vilallonga, 1968). Because

† This work was supported in part by Program Project Grant HL-240075 from the U.S. Public Health Service and by a special fellowship from the American Heart Association.
* Address correspondence to this author at the Cardiovascular Research Institute.
‡ Cardiovascular Research Institute.
§ Department of Pediatrics.
∥ Department of Anatomy.
⊥ Cancer Research Institute.
California Biotechnology, Inc.

[1] Abbreviations: SP28–36, surfactant-specific proteins with monomeric molecular weights of 28 000–36 000; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine; surfactant, surface-active material; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; PG, phosphatidylglycerol; CF, carboxyfluorescein; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; LIS, lithium diiodosalicylate.

0006-2960/87/0426-7986$01.50/0