JOURNAL OF VIROLOGY, Mar. 1977, p. 843-848
Vol. 21, No. 3

Printed in U.S.A.

Protein Kinase and Phosphoproteins of Avian Myeloblastosis Virus

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Received for publication 18 June 1976

A protein kinase associated with purified virions of avian myeloblastosis virus, BAI strain A, was highly purified by ion-exchange chromatography and gel filtration. On the basis of molecular sieving on Sephadex G-200, the enzyme protein appeared to have a molecular weight of about 50,000 to 60,000; disc gel electrophoresis in sodium dodecyl sulfate-acrylamide gels revealed the presence of at least two polypeptide chains; and isoelectric focusing on acrylamide gels revealed two protein bands with activity. Of the nonviral proteins used as phosphate acceptors, the greatest rate of phosphorylation was obtained with α-casein. Potential physiological substrates for this activity included specific virion polypeptide of avian myeloblastosis virus. One of the virion polypeptides found in association with reverse transcriptase activity from avian myeloblastosis virus accepted more phosphate than any of nonviral or viral polypeptides examined on the basis of nanomoles of ^{32}P incorporated per milligram of protein.

Investigators in recent years have described the occurrence of protein kinase in a variety of enveloped animal viruses (4-6, 8, 9, 11-15, 18-24, 26). Mention has been made of the association of this activity with purified avian myeloblastosis virus (AMV) (18). These virion-associated enzymes catalyze the in vitro phosphorylation of specific virion polypeptides (6, 9, 11, 12, 18, 19, 21, 26) and display activity as well with a variety of nonviral proteins such as α-casein, bovine serum albumin, histones, phosvitin, and protamine (6, 9, 11, 19, 23). Highly purified protein kinase preparations have been obtained from vaccinia virus (8, 9) and frog virus 3 (FV3) (19). The FV3 enzyme has been further characterized as a virus-specified component (20). Also, separation of the protein kinase activity of vesicular stomatitis virus from all of the five predominant virion structural polypeptides has been reported (6). The present study describes the purification of the AMV protein kinase, along with several characteristics of the enzyme and the reaction it catalyzes. As will be discussed, the presence of protein kinase in AMV virions and its isolation and characterization may be of much practical as well as basic significance.

MATERIALS AND METHODS

Virus. AMV, BAI strain A, was obtained from plasma of leukemic chicks maintained in this laboratory under National Cancer Institute contract 1-CP-33291. Blood was drawn into heparin by heart puncture of chicks with myeloblastosis and spun twice at 3,000 × *g* for 10 min to remove cellular material. The plasma was then sedimented at 59,000 × *g* for 40 min. The pelleted virus was resuspended and then homogenized with eight to ten strokes of a loose-fitting pestle in a tissue grinder. The suspension containing the virus was centrifuged at 3,000 × *g* for 10 min. The resulting pellet was rehomogenized and spun again at 3,000 × *g* for 10 min to wash trapped virus out of the fibrin pellet. The pooled supernatant fractions were sedimented at 59,000 × *g* for 40 min to yield the virus pellet. This cycle of low- and high-speed centrifugation was repeated twice more. The final pellet was suspended in either 10 mM Tris-chloride (pH 8.3) or 10 mM potassium phosphate (pH 7.2) in 0.15 M NaCl-1 mM EDTA, and virus concentration was adjusted to 0.02 to 0.05 g per ml (wet weight) as estimated from ATPase activity (1).

Enzyme preparations. Virus preparations, buffered either with 10 mM potassium phosphate (pH 7.2) or 10 mM Tris-chloride (pH 8.3) in 0.15 M NaCl-1 mM EDTA were lysed according to published procedures (7). RNA-dependent DNA polymerase, (RDDP) was purified essentially by the method of Kacian and Spiegelman (7), with the exception that the final step of purification employed Sephadex G-200 instead of glycerol gradient. The major peak of RDDP activity from phosphocellulose, after Sephadex G-200 gel filtration (elution buffer: 0.2 M potassium phosphate, pH 7.2, 2 mM dithiothreitol [DTT], 0.2% Triton X-100, and 10% glycerol), was used in this study, unless otherwise specified. This is also the RDDP fraction maintained in this laboratory for distribution to other investigators. After screening all the available RDDP fractions for the presence of protein kinase, DEAE-cel-

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lulose (DE-52) nonbound proteins used to purify the protein kinase served as the source of further purification by the procedure of Silberstein and August (19).

Enzyme assays. RDDP activity was measured as acid-insoluble radioactivity formed from radioactive XTP directed by the primer template as indicated. Unless otherwise noted, the reaction mixture contained 50 mM Tris-chloride (pH 8.3), 10 mM DTT, 6 mM $MgCl_2$, 40 mM KCl, 100 μg of bovine serum albumin per ml, 0.5 mM [^{3}H]NTP (10 to 20 cpm/pmol), and 0.2 mM poly(A)$_n$·poly(dT$_{12-18}$) or poly(C)$_n$·poly(dG$_{12-18}$), (the homopolymers had molar ratios of 10 adenosine to 1 thymine and 10 cytidine to 1 deoxyguanosine, respectively). Reactions (100 μl) in the presence of enzyme were incubated at 35°C, and reaction kinetics were followed by taking samples at various times onto GF/C filters and processing for acid-insoluble product (2). Unless otherwise noted, phosphorylation of substrate protein was carried out as described (18, 19) in a total incubation volume of 100 μl containing various amounts of AMV protein kinase and substrate proteins as indicated in the figure legends, 25 mM Tris-chloride (pH 8.3), 20 mM $MgCl_2$, 10 mM DTT, 20 mM KCl, 0.02% Triton X-100, and 0.2 mM [γ-^{32}P]ATP. The reaction was carried out at 35°C, and reaction kinetics were followed by taking samples on GF/C filters as described above for the RDDP assay. Unless otherwise noted, dephosphorylation of substrate protein was carried out as described (18) in a total volume of 100 μl containing various amounts of AMV phosphoprotein phosphatase (PPPase) and substrate ^{32}P-labeled proteins as indicated in the figure legends, 5 mM Tris-chloride (pH 7.5), and 0.1 mM $MnCl_2$. The reaction was carried out at 35°C, and reaction kinetics were followed by taking samples on GF/C filters as described above for the RDDP assay. Dephosphorylation with bacterial alkaline phosphatase was carried out as described above, except that the reaction mixture contained 50 mM Tris-chloride (pH 8.3) and 5 mM $MgCl_2$.

Column chromatography. Phosphocellulose (P-11) and DEAE-cellulose (DE-52) were washed and regenerated as previously described (25).

Gel filtration. Sephadex G-200 (Pharmacia) was allowed to swell in the buffers designated above, and fine particles were removed by decantation. The Sephadex was packed into appropriately sized columns and equilibrated with the buffer used for elution as designated above.

Gel electrophoresis. Electrophoresis in the presence of 0.1% sodium dodecyl sulfate was carried out in an apparatus manufactured by E-C Apparatus Co. (model 458) using columns (6 by 120 mm) of 15% polyacrylamide separating gel and using a 6% polyacrylamide stacking gel as previously described (10). Protein samples were prepared and applied as previously described (10). One set of gels was stained in a 0.05% solution of Coomassie brilliant blue R-250 and photographed as described (3). Gels for measuring ^{32}P-radioactivity distribution were immediately frozen, sliced into 1-mm sections, and solubilized, and radioactivity distribution was determined (18). The molecular weight of AMV polypeptides was estimated from their migration relative to proteins of known molecular weight (17).

Isoelectric focusing on acrylamide gels. Isoelectric focusing was carried out on columns (6 by 80 mm) of 7.5% polyacrylamide gels containing 0.2% ampholine in the pH ranges of 3.5 to 10 and 5 to 8 as previously described (25). One set of gels was immediately frozen, sliced into 1-mm sections, and polypeptides were extracted with 100 mM potassium phosphate (pH 7.2), 10 mM DTT, 0.2% Triton X-100, 10% glycerol, and 1 mg of bovine serum album per ml. The gel slices in extraction buffer were frozen and thawed three times and allowed to extract at 4°C overnight. RDDP, protein kinase activities, and ^{32}P radioactivity distributions were determined as described above.

Chemicals. Synthetic polyribonucleotides, polydeoxyribonucleotides, and oligodeoxyribonucleotides were obtained from P-L Biochemicals and/or Collaborative Research, Inc.; [^{3}H]TTP (47 Ci/mmol), [^{3}H]dGTP (13 C/mmol) and [^{3}H]-poly(A) (8.5 mCi/mmol of polynucleotide phosphorus) were obtained from Schwarz/Mann and dried down to remove ethyl alcohol before use. Poly(A) was purchased from Miles Laboratories; [γ-^{32}P]ATP (10 to 30 Ci/mmol) was obtained from New England Nuclear. Bacterial alkaline phosphatase (free of ribonuclease activity) was purchased from Worthington Biochemical Co. Whatman DEAE-cellulose (DE-52), phosphocellulose (P-11), and GF/C paper for filter disks came from Reeve Angel; Sephadex G-200 and blue dextran were obtained from Sigma Chemical Co. All reagents for disc gel electrophoresis were from E-C Apparatus Corp.; ampholine carrier ampholytes were purchased from LKB Instruments Inc., and enzyme-grade sucrose, urea, ammonium sulfate, and molecular weight markers were from Schwarz/Mann Bio Research.

RESULTS

Detection of protein kinase. In an effort to increase the yield and improve the quality of RDDP from AMV, systematic alterations of the isolation procedures have been undertaken (C. M. Tsiapalis, G. H. Houts, and J. W. Beard, Nucleic Acids Res., no. 3, p. 2267, 1976). On the basis of the observation that AMV-RDDP activity is found associated with a previously unreported small acidic protein appearing even in preparations of highly purified enzyme (C. M. Tsiapalis, Nature [London], in press) and the report on the observation of RSV-RDDP phosphorylation in vitro (11), it was necessary to find, isolate, and characterize the enzyme involved in this phosphorylation reaction. There was no problem detecting a protein kinase-like activity in purified AMV. The results of such an experiment, with both nonviral and viral proteins used as phosphate acceptors, are presented in Fig. 1B. Comparison of the amount of protein kinase between AMV (Fig. 1C) and FV3 (Fig. 1B) confirms the high levels of pro-

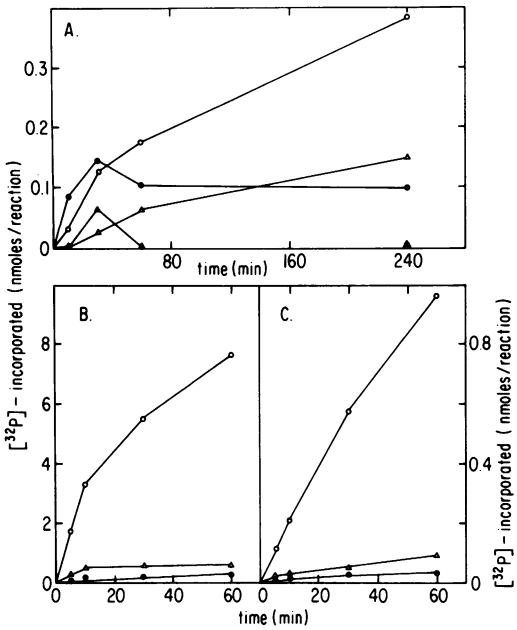

FIG. 1. *(A) Incorporation of radioactivity from [γ-32P]ATP by purified AMV protein kinase in the presence of various AMV-RDDP fractions. Each reaction mixture (0.1 ml), at the same concentration of reaction components as described in Materials and Methods, contained 5 μl of the concentrated phosphocellulose fraction of protein kinase and concentrations of AMV-RDDP fractions per reaction as follows: Symbols: ○, 14 μg of DE-52 fraction; ●, 55 μg of phosphocellulose fraction; △, 4.6 μg of G-200 fraction; ▲, 8 μg of the load and wash from phosphocellulose. (B and C) Incorporation of radioactivity from [γ-32P]ATP by FV3 and AMV protein kinases in the presence of nonviral and viral phosphate acceptor proteins. Each reaction mixture (0.1 ml), at the same concentrations of reaction components as described in Materials and Methods, contained (B) 2 μl of FV3 (×100) and (C) 20 μl of AMV (0.05 g/ml). The amounts of phosphate acceptor protein per reaction were as follows: ○, 4 mg of α-casein; ●, 4 mg of protamine sulfate; and △, 0.2 mg of partially purified AMV-RDDP.*

tein kinase activity found in the latter (19) and illustrates that AMV, like FV3 (19), recognizes acidic phosphoproteins (i.e., α-casein) better than basic phosphoprotein (i.e., protamine) in contrast to the poxvirus activity (8, 9). In addition, in the course of these studies a PPPase activity was detected (Fig. 1A), and its isolation and characterization will be reported elsewhere. Screening of all available AMV-RDDP fractions in this laboratory immediately afforded recognition of the protein kinase distribution, and this allowed its separation from RDDP and partial characterization as described below.

Purification of protein kinase. In the process of RDDP purification, after lysis of purified AMV at 4°C in the presence of high salt and detergent, the lysate upon appropriate dilution is loaded onto DE-52 (7). Under these conditions, about 5 to 10% of the total solubilized protein from the lysate binds to DE-52, including 80 to 90% of the RDDP activity, whereas most of the protein kinase does not bind to DE-52. Fractionation of the DE-52 nonbound proteins on phosphocellulose (P-11, 2.6 by 10 cm) essentially as previously described (7) is presented in Fig. 2. The proteins bound to phosphocellulose were eluted with a 20-mM to 800-mM KCl gradient, and a 180-ml total volume of buffer A was used to wash the column (10 mM Tris-chloride, pH 7.5, 20% glycerol, 0.2% Triton X-100, and 2 mM DTT). Finally, the column was washed with 50 ml of the above buffer containing 0.8 M KCl, and 2-ml fractions were collected. Every other fraction was assayed for RDDP and protein kinase. Elution with this gradient effected only limited separation of these two activities. The fractions containing most of the protein kinase activity (46 to 80 = 50 ml), after 1:10 dilution in buffer A, was loaded onto a small phosphocellulose (P-11, 1.5 by 3 cm), the column was washed with buffer, and activities were eluted with buffer containing 1 M KCl. This concentrated the enzyme solution about 20-fold. In the presence of blue dextran, aldolase, and myoglobin, 2 ml of the above concentrated fraction made 20% in glycerol was loaded onto Sephadex G-200 (1.6 by 60

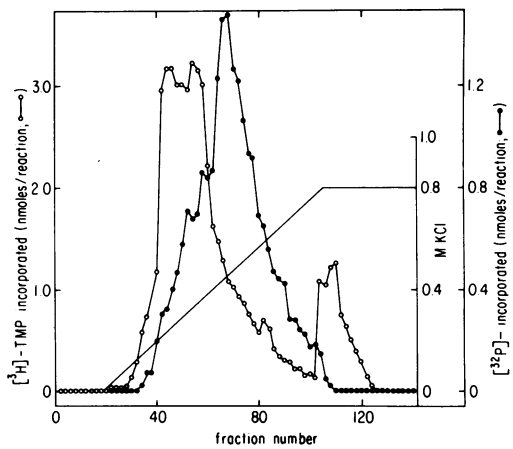

FIG. 2. *Purification of protein kinase by gradient elution chromatography on phosphocellulose. Fractionation was carried out as described in the text. After fractionation, 10 μl of every other fraction was assayed for protein kinase and RDDP, each in a 60-μl final reaction volume. Incubation was carried out for 60 min for protein kinase and 10 min for RDDP at 35°C. Symbols: ○, RDDP activity; ●, protein kinase activity.*

cm) preequilibrated with buffer A in 1 M KCl, and 0.75-ml fractions were collected. Every other fraction was assayed for RDDP and protein kinase activities. The results are shown in Fig. 3. This step affords good separation of protein kinase from RDDP. The protein kinase active pool from G-200 was immediately concentrated for storage as described above. Unless otherwise specified, this was the protein kinase fraction used in this study. The RDDP fractions were used for further studies, as will be reported elsewhere.

Purity of enzyme. A major band coinciding with the activity was found after isoelectric focusing of the concentrated G-200 fraction, although some splitting of the major protein band was observed with enzyme activity found in both bands when the gel was sliced, extracted, and analyzed for enzyme activity (results not shown). Electrophoresis of the purified preparations in 0.1% sodium dodecyl sulfate on 15% acrylamide gels showed two major bands of protein in these preparations with the addition of several minor protein bands (data not shown). These results indicate that the enzyme needs additional purification before attempts can be made to identify the polypeptide(s) responsible for the activity.

Nonviral and viral phosphate acceptors. Of all the nonviral proteins used as phosphate acceptors here, α-casein was found to be the best phosphate acceptor (Table 1; Fig. 4). Thus, AMV and FV3 (19), both highly purified protein kinases, displayed specificity for acidic phosphate acceptor proteins as substrates. Furthermore, Table 1 shows that potential physiological substrates for this activity include virion polypeptides of AMV. Fractionation of the in vitro phosphorylated AMV polypeptides from the DE-52 pool of RDDP on Sephadex G-200 revealed the separation of the phosphorylated polypeptides into several species (Fig. 5). One of the phosphorylated polypeptides was found to elute in the same fractions as the RDDP activity. A small phosphoprotein was found to elute behind myoglobin, and the other was essentially excluded from the column.

TABLE 1. *Specificity of action of the avian myeloblastosis virus protein kinase*[a]

Phosphate acceptors	^{32}P radioactivity incorporated		
	mg/reaction	nmol/reaction in 30 min	nmol/mg of protein in 30 min
Protamine sulfate	2.500	1.95	0.78
α-Casein	2.500	48.78	19.51
AMV-RDPP[b]	0.200	2.75	13.75
AMV-RDDP[b] + α-casein	2.700	18.92	7.00
AMV-RDDP[c]	0.007	0.25	35.71
AMV-RDDP[c] + α-casein	2.007	13.13	6.54

[a] Details of protein kinase reaction are given in the legend to Fig. 4.
[b] Partially purified fraction of enzyme.
[c] Purified fraction of enzyme.

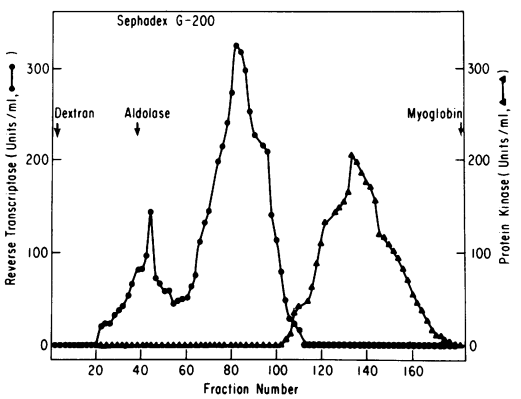

FIG. 3. *Final purification of protein kinase by molecular sieving on Sephadex G-200. Fractionation was carried out as described in the text. After fractionation, 2 μl of every other fraction was assayed for protein kinase and RDDP, each in 27-μl final reaction volumes. Incubation was carried out for 10 min for both RDDP and protein kinase at 35°C. In a separate experiment, the elution positions of aldolase was determined by absorbance at 280 mm, and that of blue dextran was determined by absorbance at 407 mm (indicated by arrows). Symbols: ●, RDDP activity; ▲, protein kinase.*

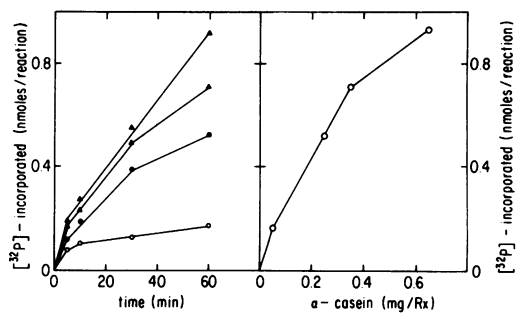

FIG. 4. *Kinetics of α-casein phosphorylation at several concentrations of α-casein. Each reaction mixture (0.1 ml), at the same concentrations of reaction components as described in Materials and Methods, contained 2 μl of the concentration G-200 fraction of protein kinase and concentrations of α-casein per reaction as indicated below. The [γ-^{32}P]-ATP contained 85 cpm per pmol. Samples (20 μl) were removed at intervals, and the incorporation of ^{32}P was measured as described. Symbols: ○, 50 μg; ●, 250 μg; △, 375 μg; ▲, 625 μg.*

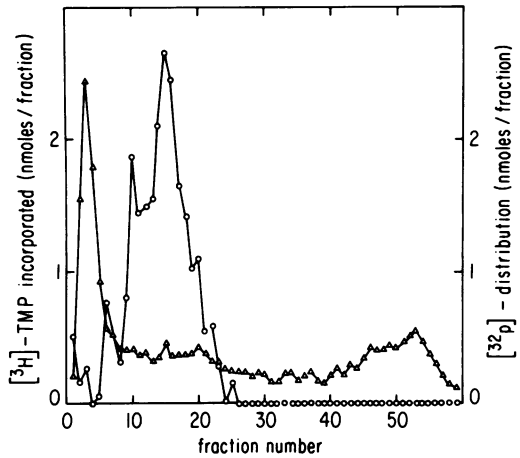

FIG. 5. *Chromatographic profiles on Sephadex G-200 of partially purified AMV-RDDP activity and ^{32}P radioactivity after phosphorylation with AMV protein kinase for 30 min at 35°C. At the completion of the protein kinase as described in Materials and Methods, all reaction products were dialyzed against buffer A (200 mM potassium phosphate [pH 7.2]-0.1 mM EDTA-2 mM DTT-0.2% Triton X-100 containing 10% glycerol) for 36 h with four changes of dialysis buffer to remove [α-^{32}P]rATP. Portions (0.5 ml; one-fourth of the protein kinase reaction) were subjected to chromatography by molecular sieving on Sephadex G-200 (1.6 by 60 cm) equilibrated with buffer A. Fractions (1.8 ml) were collected and analyzed for ^{32}P radioactivity (△) as described in Materials and Methods. RDDP activity (○) was determined after incubation of 10-μl samples for 60 min at 35°C with 50 μl of RDDP standard reaction mixture; 50-μl samples from each reaction were placed on GF/C disks and worked up as described (2).*

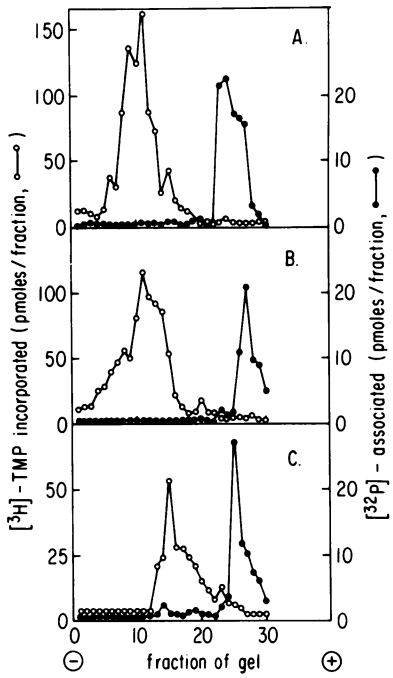

FIG. 6. *Isoelectric focusing on in vitro phosphorylated AMV-RDDP fractions. Electrophoresis conditions, staining of gels for proteins, extraction of gel slices for enzyme activity, ^{32}P distribution, and assays for enzyme activity and radioactivity in gel extracts are described in Materials and Methods. (A) 8.6 μg of a concentrated G-200 RDDP fraction, (B) 27 μg of a concentrated phosphocellulose fraction and (C) 43 μg of a concentrated fraction from the load and wash of phosphocellulose were connected in tandem with DE-52.*

Fractionation of the most purified AMV-RDDP (inventory enzyme) after in vitro phosphorylation on G-200 revealed the elimination of the largest phosphoprotein(s) observed above (C. M. Tsiapalis, Nature [London], in press). Upon isoelectric focusing of the phosphoprotein fraction associated with RDDP (Fig. 5, fractions 12 to 24), essentially complete dissociation of the phosphoprotein from RDDP was revealed (Fig. 6). The physicochemical properties of this small acidic phosphoprotein (designated by φ) and its influence on RDDP-catalyzed DNA synthesis in vitro will be reported elsewhere (Tsiapalis, Nature [London], in press; manuscript in preparation). On the basis of nanomoles of ^{32}P incorporated per milligram of protein (Table 1), φ seems to display the highest specific activity of all the nonviral and viral polypeptides examined.

DISCUSSION

Many enveloped animal viruses have been found to display protein kinase activity (4–6, 8, 12–15, 18–21, 23, 24, 26). Highly purified enzyme has been only reported from poxvirus (8, 9) and FV3 (19). In the case of FV3, characterization of protein kinase as a virus-specified component has also been reported (20). Silberstein and August have meticulously compared the physicochemical properties of this activity from poxvirus and FV3 (19). Furthermore, on the basis of their detail study of the FV3 protein kinase, they were able to see its resemblance to other protein kinases such as casein kinase, phosvitin kinase, and others (for review, see references 19 and 22). No evidence, however, is presently available concerning the biology of protein kinase in virion polypeptide phosphorylation.

This paper reports the isolation of highly purified protein kinase from AMV utilizing a large-scale method that is also suitable for purification of RDDP, PPPase, and other AMV polypeptide(s) (to be reported elsewhere). After removal of most RDDP on DE-52-cellulose at

pH 7.2, molecular sieving on Sephadex G-200 removes the remaining RDDP. Chromatography on phosphocellulose removes a PPPase. The results reported here on the physicochemical properties of the protein kinase activity from virions of AMV indicate similarities to those of FV3 protein kinase (19). Although further detail studies on this enzyme activity are in order, the protein kinase used in this study has permitted phosphorylation in vitro of specific AMV polypeptides. One of these polypeptides, ϕ, found in partially AMV-RDDP as well as with our most purified RDDP fraction, has been shown to accept more phosphate than any of nonviral or viral polypeptides examined on the basis of nanomoles of ^{32}P incorporated per milligram of protein. Isolation of highly purified protein kinase will not settle the biological role of protein phosphorylation in enveloped animal viruses, but it does provide a means for further studies on the nature and requirements of physiological phosphate acceptor polypeptides, the characterization of the enzyme products, mechanism of enzyme action, and enzymic modification of phosphate acceptor polypeptides of interest.

ACKNOWLEDGMENTS

I thank M. Gravell for providing FV3.

This work was supported by Public Health Service contract NO1 CP 33291 within the Virus Cancer Program of the National Cancer Institute.

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