Protein Kinase Activity in Purified Poliovirus Particles and Empty Viral Capsid Preparations

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SUMMARY

Preparations of purified poliovirus type 1, strain Mahoney, and empty viral capsids contain a protein kinase activity. The γ-phosphoryl group of $[^{32}\text{P}]\text{ATP}$ is transferred to all of the capsid proteins. Viral proteins phosphorylated in vitro are recognized by antiserum directed against isolated viral capsid proteins, indicating that phosphorylation does not alter the antigenic sites to such an extent that the recognition by antibodies is abolished. Viral capsid protein phosphorylation exposes new antigenic sites and leads to a destabilization of the virions. The transfer of $^{32}\text{P}$ to viral proteins is linear for 90 min at 37 °C in the presence of 10 mM-Mg$^{2+}$ at pH 8.1. Reducing agents or virus-stabilizing agents (such as arildone) reduce the kinase activity and result in a different pattern of capsid protein phosphorylation.

INTRODUCTION

The poliovirus capsid is composed of 60 copies of VP$_1$ and VP$_3$, 58 or 59 copies of VP$_2$ and VP$_4$, and 1 or 2 copies of VP$_0$, the precursor of VP$_2$ and VP$_4$ (for review, see Rueckert, 1976). Incubation of virions at pH 10.5 (Van Elen & Boeye, 1966) or at 56 °C (Breindl, 1971) results in release of VP$_4$ and conversion of the virions into empty capsids. The native poliovirus capsid is stable in 1% SDS (Fenwick & Wall, 1973) at room temperature. Complete dissociation of the viral capsid into its protein components is routinely obtained by boiling the virus preparation in 1% SDS. Recently, antisera to isolated viral capsid proteins have been prepared and described. Only anti-VP$_2$ serum reacts with intact virions (Meloen et al., 1979).

Protein kinases or protein phosphotransferases (EC 2.7.1.37) and phosphoprotein phosphohydrolases (EC 3.1.3.16) are involved in the regulation of many biochemical pathways, notably in cell proliferation and in the initiation of protein synthesis (for review, see Rosen & Krebs, 1981). In addition, protein kinases have been detected in a number of enveloped viruses, such as Rauscher murine leukaemia virus (Strand & August, 1971), Semliki Forest and Sindbis viruses (Tan & Sokol, 1974), herpes simplex virus (Lemaster & Roizman, 1980), vaccinia virus (Downer et al., 1973), and vesicular stomatitis virus (Witt & Summers, 1980). More recently kinase activity has been found associated with a few non-enveloped viruses [foot-and-mouth disease virus (Grubman et al., 1981); adenovirus (Akusjarvi et al., 1978)].

Nevertheless, the origin of most of the virus-associated protein kinases is obscure. Evidence that these kinases are coded for by viral genes has been found in frog virus 3 (Silberstein & August, 1973) and herpes simplex virus (Blue & Stubbs, 1981), while Akusjarvi et al. (1978) suggest that the adenovirus type 2-associated protein kinase might be of host origin. In this paper, we report that a protein kinase activity is associated with purified poliovirions which phosphorylates viral structural proteins and leads to a destabilization of the virions and to exposure of new antigenic sites. Some properties of the enzyme are described. A preliminary report of these findings has been published (Schärli & Koch, 1981).

METHODS

Virus and cells. HeLa S3 cells were grown in suspension culture in minimal essential medium (MEM, Joklik's modification) supplemented with 7.5% foetal calf serum (FCS, Boehringer). Poliovirus type 1 (strain Mahoney or Sabin LSc2ab) was used in all experiments.
RESULTS

Identification of proteins phosphorylated in an endogenous poliovirus phosphotransferase reaction

Incubation of highly purified poliovirus particles in complete reaction mixture in the presence of \([\gamma-\text{32P}]\text{ATP}\) for 90 min at 37 °C resulted in the incorporation of \(\text{32P}\) into virus particles. In order to identify phosphorylated proteins, the virions were dissociated and analysed by SDS–PAGE. The individual viral proteins in the gel were visualized by staining with Coomassie Brilliant Blue (Fig. 1, lane a). The major capsid proteins VP1, VP2, and VP3 are clearly visible. The corresponding autoradiograph (Fig. 1, lane b) reveals that not only were VP1, VP2, and VP3 phosphorylated, but also the minor capsid component VP0. The position of VP0 is indicated in Fig. 1 lane (c), which shows the protein composition of \(\text{35S}\)-labelled empty capsids run in parallel. In addition to the viral capsid proteins, other phosphorylated proteins migrated between VP0 and VP1 in the gel (Fig. 1, lane b). The nature of these labelled proteins is not clear. We designate these proteins Pz.

In further experiments we analysed the antigenic properties of the phosphorylated viral proteins. Protein phosphorylation was performed as before and the reaction was stopped by transfer of the tube to an ice-bath. Anti-VP2 serum (the only one that reacts with the complete virus particle (Meloen et al., 1979)) was added and immunoprecipitation was performed. The
VP2 precipitate was analysed by PAGE. The protein pattern (Fig. 2b) is identical to that of the control (Fig. 2a), i.e. the phosphorylated but not immunoprecipitated virus. The proteins Pz were co-precipitated with the anti-VP2 serum, indicating that they are part of the virus or are virus-associated.

However, Pz proteins did not react with anti-VP2 serum nor with anti-VP1 and anti-VP3 sera (data not shown) when the phosphorylated virus was disassembled by boiling in 1% SDS prior to the addition of the individual antisera. Under these conditions, anti-VP2 serum reacted mainly with VP0 and VP2 (Fig. 2, lane c). It appears unlikely that Pz proteins are related to the poliovirus capsid proteins.

**Kinetics of protein phosphorylation**

The kinetics of protein phosphorylation was assayed at 30 °C and 37 °C (Fig. 3). The autoradiograph of the SDS gel was evaluated by densitometry and integration of the areas under the peaks. At 37 °C the total incorporation of 32P was linear up to 90 min (Fig. 3c). However, the labelling of individual viral capsid proteins takes place in succession. On the other hand, at 30 °C, significant 32P labelling was seen only after 90 min incubation (Fig. 3b). The first protein to be phosphorylated was VP2 followed by VP1, VP0 and Pz and then VP3 (Fig. 3a). This result has been confirmed in three independent experiments. At 30 °C the proteins in the capsid of the virus particles are tightly packed and either the kinase is hidden or the potential
phosphorylation sites on the virions are not accessible. Since the phosphorylation of viral proteins leads to destabilization of the virions, the kinase reaction takes place as soon as a critical number of viral proteins are phosphorylated. Again, as at 37 °C, the first viral protein to be phosphorylated is VP2 and the last is VP3. In this regard, it is of interest to note that the relative degree of phosphorylation of VP3 increases threefold when the phosphorylation reaction is carried out at 56 °C. Also, in naturally occurring empty capsids, VP3 is preferentially phosphorylated. To test for phosphate exchange and phosphatase activity, poliovirus particles labelled with $^{32}$P by a 90 min labelling period at 37 °C were further incubated for 60 min with an excess of unlabelled ATP (1 mM). The amount of $^{32}$P already incorporated into capsid proteins was not reduced under these conditions (data not shown), indicating that phosphate exchange does not occur.

Properties and requirements of the endogenous poliovirus phosphotransferase reaction

Most cellular and viral protein kinases have been assayed in the presence of Mg$^{2+}$. The poliovirus-associated protein kinase also requires MgCl$_2$; no activity was seen in the absence of Mg$^{2+}$, and optimal kinase activity was observed at 10 mM-Mg$^{2+}$. At 5 mM- or 15 mM-MgCl$_2$, the transfer of $^{32}$P to VP$_2$ was 58% and 46% respectively of the amount of $^{32}$P incorporated in the presence of 10 mM-MgCl$_2$. The pH range of the enzyme activity was rather broad, with an optimum around pH 8 (data not shown). The enzyme was fully active after treatment of the virions for 10 min at 56 °C (see below). Exposure of poliovirus to 95 °C for 5 min destroyed the kinase activity.

Many protein kinases isolated from animal cells have been reported to be stimulated by cyclic
Fig. 3. $^{32}P$ incorporation in poliovirus capsid proteins at 37 °C and 30 °C. The kinase assay (as described in Methods) was performed at 37 °C (a) or at 30 °C (b). At the indicated times (min), samples were taken and prepared for SDS gel electrophoresis. The stained and dried gels were autoradiographed. The densitometer scans of the films after 3 days exposure are shown. (c) Sum of the integrated peak areas of the experiment performed at 37 °C (a).

Table 1. Relative rates of phosphorylation of poliovirus capsid proteins*

<table>
<thead>
<tr>
<th></th>
<th>+ DTT</th>
<th>+ Cys</th>
<th>+ 2-ME</th>
<th>+ Arildone</th>
<th>+ cAMP</th>
</tr>
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<tr>
<td>VP0</td>
<td>100</td>
<td>61</td>
<td>45</td>
<td>105</td>
<td>36</td>
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<tr>
<td>VP1</td>
<td>100</td>
<td>63</td>
<td>60</td>
<td>373</td>
<td>16</td>
</tr>
<tr>
<td>VP2</td>
<td>100</td>
<td>51</td>
<td>11</td>
<td>ND</td>
<td>58</td>
</tr>
<tr>
<td>VP3</td>
<td>100</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>90</td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
<td>60</td>
<td>60</td>
<td>373</td>
<td>16</td>
</tr>
</tbody>
</table>

* Unless indicated otherwise, purified poliovirus was incubated in complete kinase reaction mixture at 37 °C (control). After 90 min the reactions were stopped by the addition of gel sample buffer (Laemmli, 1970). Gels were run as described in Methods, and the dried gel was autoradiographed. The film was scanned in an Ortec scanner. The integrated peak areas of each individual protein band are expressed as a percentage of the corresponding peak in the parallel control experiment.

† ND, Not detectable.

nucleotides (for review, see Krebs & Beavo, 1979). We performed the phosphotransferase assays in the presence of 1 mM-cAMP. Although it had no stimulatory effect on the poliovirus-associated kinase (Table 1), the presence of cAMP changed the phosphorylation pattern: VP0, VP1, and VP2 carried only small amounts of $^{32}P$, but VP3 was highly phosphorylated.

**Phosphorylation of poliovirus capsid proteins in the presence of reducing agents**

Most virus-associated protein kinases have been reported to be more active in the presence of dithiothreitol (DTT). The poliovirus-associated protein kinase did not require DTT: in contrast, its activity was inhibited, and the amount of $^{32}P$ incorporated in each individual capsid protein was reduced to a different degree in the presence of 10 mM-DTT (Table 1). We therefore tested the effect of other sulphydryl reagents on the kinase activity. As shown in Table 1, the
Fig. 4. Immunoprecipitation of poliovirus. Purified poliovirus preparations, either unlabelled (lanes a to c), labelled in vivo with \[^{35}S\]methionine (lanes d to g), or labelled in vitro with \(^{32}P\) (lanes h to k) as described in Methods, were immunoprecipitated with antiserum prepared against isolated and purified viral capsid proteins by adsorption to S. aureus as described by Racevskis & Koch (1978). VP\(_3\) was weakly phosphorylated in this experiment. In (a), (d) and (h) anti-VP1, in (b), (e) and (i) anti-VP2 and in (c) and (f) anti-VP3 serum was added to the virus preparation. SDS electrophoresis of the precipitates was performed following the method of Laemmli (1978). The gel was stained with Coomassie Brilliant Blue (lanes a to c). Autoradiographs were obtained after a 3 day exposure on Kodak X-Omat R film (lanes d to k). Lane (g), empty capsids labelled in vivo with \[^{35}S\]S included for reference; lane (k), phosphorylated in vitro non-immunoprecipitated poliovirus (an aliquot of which was immunoprecipitated in h and i); sp, serum proteins; df, dye front.

presence of 20 mM-cysteine inhibited the transfer of \(^{32}P\) to the individual capsid proteins to a similar degree as 10 mM-DTT. In the presence of 1\(^\%\) 2-mercaptoethanol (2-ME), VP\(_0\) and VP\(_1\) were highly phosphorylated but the transfer of \(^{32}P\) to VP\(_2\) and VP\(_3\) seemed to be completely inhibited (Table 1).

Phosphorylation of natural and artificial empty capsids

In order to localize the enzyme we performed the following experiments. We incubated poliovirus particles for 10 min at 56 °C (a treatment which produces empty virus shells) and then performed the kinase assay at 37 °C for 90 min; the protein kinase was still fully active. When the kinase assay was performed at 56 °C, the phosphorylation pattern of the individual capsid proteins was changed (Table 1). The capsid protein VP\(_0\) was no longer phosphorylated and more label was incorporated into VP\(_3\) compared to the control at 37 °C.

These experiments suggest but do not prove that the kinase is still associated with the empty capsids produced in vitro. To verify that the kinase activity is associated with the viral shell, we assayed naturally occurring empty particles purified by CsCl density gradient centrifugation. As seen in Table 1, purified empty capsids also contained the kinase activity. Although the empty particles contain significantly more VP\(_0\), the amount of \(^{32}P\) incorporated in VP\(_0\) was not higher than in intact particles.

Phosphorylation-induced structural alterations of the poliovirus capsid

As demonstrated above, phosphorylation of poliovirus capsid proteins in vitro does not alter the primary antigenic reactivity of the poliovirus peptides (see Fig. 2), i.e. phosphorylated poliovirus proteins are recognized by specific antisera to the individual SDS-denatured purified capsid proteins. However, the antigenic reactivity of the viral proteins in intact virions is altered after phosphorylation. As demonstrated in Fig. 4 (lanes a to g), only anti-VP\(_2\) serum reacted with intact poliovirus (Meloen et al., 1979) whereas after the phosphorylation reaction all three antisera tested (anti-VP\(_1\), anti-VP\(_2\) and anti-VP\(_3\)) were able to immunoprecipitate the virus. The gel pattern obtained by SDS-PAGE analysis of the immunoprecipitated virus (Fig. 4, lanes h and i) was identical to the one obtained with the phosphorylated but not immunoprecipitated
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Fig. 5. Gel electrophoresis of poliovirus. Purified poliovirus was labelled *in vitro* with $^{32}$P as described in Methods. Before separation of the viral polypeptides by SDS–PAGE (according to Laemmli, 1970) the sample in SDS sample buffer (Laemmli, 1970) was divided: (b) and (d) are the unboiled aliquots; (a), (c) and (e) were boiled for 5 min prior to application on the gel. (a to c) Coomassie Brilliant Blue-stained proteins; (d and e) autoradiograph of $^{32}$P-labelled poliovirus.

Table 2. Immunoprecipitation of phosphorylated poliovirus capsid proteins*

<table>
<thead>
<tr>
<th>Immunoprecipitation performed with</th>
<th>anti-VP₁</th>
<th>anti-VP₂</th>
<th>anti-VP₃</th>
</tr>
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<tbody>
<tr>
<td>VP₀</td>
<td>93</td>
<td>100</td>
<td>106</td>
</tr>
<tr>
<td>VP₁</td>
<td>112</td>
<td>100</td>
<td>91</td>
</tr>
<tr>
<td>VP₂</td>
<td>104</td>
<td>100</td>
<td>96</td>
</tr>
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</table>

* Phosphorylation *in vitro* of poliovirus was performed as described in Methods. Aliquots of the reaction mixture were diluted with PBS, and 10 μl of the corresponding antiserum was added. Immunoprecipitation was carried out as described by Racevskis and Koch (1978). The precipitates were separated by SDS gel electrophoresis, and the radioactive bands were visualized by autoradiography. The film was scanned in an Ortec scanner and the peak areas were expressed in arbitrary units. The anti-VP₂ precipitate was taken as 100%. VP₃ was not evaluated in this experiment.

Each individual serum gave rise to a gel pattern showing all three phosphorylated capsid proteins in equal amounts (Table 2).

**Changes in the stability of the phosphorylated poliovirus capsid**

The partial unfolding of the capsid structure, which was indicated by the exposure of new antigenic sites, did not lead to a complete dissociation of the capsid structure. As seen in Fig. 4...
(lane k), at least a subunit structure consisting of VP₀, VP₁ and VP₂ was retained. This conclusion is further supported by sucrose gradient analysis of purified [³⁵S]methionine-labelled poliovirus incubated at 37 °C with or without 1 mM-ATP. In the control as well as in the ATP-treated sample, the label was found only in the position of intact virus (data not shown). We also could not find significant differences between ATP-treated and untreated virus preparations with regard to adsorption rate and infectivity. We therefore conclude that phosphorylation of poliovirus capsid proteins does not trigger disintegration of the virions. However, the sensitivity of the capsid to SDS is increased. While normal poliovirus is stable in 1% SDS gel sample buffer (Laemmli, 1970) at room temperature, phosphorylated poliovirus fell apart under these conditions. In Fig. 5 we show that phosphorylated capsid proteins enter the gel even without boiling (Fig. 5, lane d), but non-phosphorylated viral proteins do not (Fig. 5, lane b).

The antiviral drug arildone is thought to specifically prevent virus uncoating (Caliguiri et al., 1980). In the presence of arildone, the phosphorylation of the poliovirus capsid proteins was greatly reduced (Table 1). Only 36% of the [³²P] label was incorporated in VP₀, 16% in VP₁ and 58% in VP₂ in the presence of 0.3 μM-arildone compared to the amount of [³²P] incorporated in the control.

Phosphorylation in vivo of poliovirus in infected HeLa cells

Analysis of cell-released purified aphthovirus A₁₂ revealed that two of the major polypeptides, VP₄ and VP₅, were highly phosphorylated in vivo and that VP₁ and VP₂ were phosphorylated to a lesser extent (La Torre et al., 1980). We labelled poliovirus-infected HeLa cells with inorganic [³²P] from 2 to 4 h post-infection. SDS-PAGE analysis of these cells revealed several phosphorylated protein bands comparable to those described by James & Tershak (1981). In parallel we prepared extracts of these cells and performed immunoprecipitation using antisera specific to poliovirus capsid proteins and adsorption to S. aureus. On SDS-PAGE analysis of the immunoprecipitates, we could not detect phosphoproteins with poliovirus capsid antigenic specificity.

DISCUSSION

We report here on the presence of a protein kinase in several independently prepared and purified poliovirus and empty capsid preparations. The purification procedure included SDS and Triton X-100 treatment as well as exposure of purified virions to chloroform (for details see Methods). A corresponding kinase, however, was not detectable under our assay conditions in preparations of the attenuated poliovirus strain Sabin LSc2ab after growth in HeLa cells and purification in the same way as wild-type poliovirus. However, the coat proteins of LSc2ab virus particles are phosphorylated by incubation with wild-type virions (unpublished results).

The poliovirus protein-phosphotransferase conveys the γ-phosphoryl group of [³²P]ATP to the viral coat proteins VP₁, VP₂ and VP₃. The minor viral capsid protein VP₀, which is present only in one to two copies per particle (Rueckert, 1976), is heavily phosphorylated. In addition, proteins (Pz) migrating between VP₀ and VP₁ are also highly phosphorylated. They do not react with any of the antisera prepared against isolated viral capsid proteins. Since the reactivity of the isolated viral capsid proteins with specific antibodies is not lost by phosphorylation (Fig. 2), it is likely that Pz proteins are not related to one of the capsid proteins. Pz might represent non-capsid viral proteins or cellular proteins.

The individual viral proteins are phosphorylated in vitro to various extents depending on the experimental conditions (Table 1). In the presence of 10 mM-DTT only VP₀, VP₁ and VP₂ are phosphorylated while the VP₃ band is not detectable. This is in contrast to protein kinases associated with other animal viruses like Semliki Forest virus and Sindbis virus (Tan & Sokol, 1974), herpes simplex virus (Lemaster & Roizman, 1980), vaccinia virus (Downer et al., 1973) and vesicular stomatitis virus (Witt & Summers, 1980) which show enhanced kinase activity in the presence of DTT. It is known that certain reducing agents stabilize poliovirions against heat inactivation (see Lonberg-Holm & Philipson, 1974). Therefore, it is conceivable that phosphorylation sites on the virion are not accessible under reducing conditions, and destabilization of the capsid might even be a prerequisite for phosphorylation. This view is
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Supported by the time lag of capsid phosphorylation observed at 30°C (Fig. 3b) and by the inhibitory effect of arildone on phosphorylation of viral proteins. Of interest in this respect are results obtained with foot-and-mouth disease virus (FMDV, another picornavirus). Purified preparations of FMDV contain a comparable protein kinase which is active only in disrupted virus particles (Grubman et al., 1981). These results also indicate that the kinase is an integral part of the viral capsid.

In this paper we present evidence that phosphorylation of the capsid results in structural alterations of the viral shell. Protein modifications like phosphorylation may lead to structural changes of proteins due to the additional negative charges. It is thus very likely that complex structures like virus protein shells might undergo conformational changes which may result in a rearrangement of their protein(s). As demonstrated above, the poliovirus structure unfolds somewhat due to phosphorylation; this is clearly shown by the exposure of two new antigenic sites. This unfolding leads to a destabilization of the capsid which is demonstrated by an increased sensitivity to SDS.

We suggest that an opening of the structure might be a prerequisite for phosphorylation. Lonberg-Holm & Whitely (1976) suggest that early modifications destabilize the viral capsid and that these alterations are required for uncoating. Arildone as well as cysteine stabilize the capsid and thus inhibit both phosphorylation and uncoating. We propose a new role of protein phosphorylation in the uncoating mechanism: an infecting poliovirus particle enters the cell and intracellularly (at 37°C) comes in contact with ATP. The virus particle starts to open up, which leads to activation of the kinase or to an exposure of the phosphorylation sites. The phosphorylation of the capsid proteins leads to a further irreversible unfolding of the viral capsid.

The natural target protein(s) of all virus-associated protein kinases are largely unknown. Speculation concerning the functions of virion-associated kinases has arisen from the regulatory nature of phosphorylation reactions in cellular metabolism. It has been suggested that protein phosphorylation may be involved in virus–host interactions and virus-mediated modifications of host cell metabolism. Infection of tissue culture cells by polioviruses leads to a rapid inhibition of cellular macromolecular synthesis, usually referred to as 'shut-off' (for review, see Koch et al., 1982). After specific adsorption of polioviruses to receptors on the host cell membrane, approximately 50% of the viral capsid proteins are detectable intracellularly and remain there in a rather stable form (Habermehl et al., 1974). It is conceivable that viral proteins might interfere with the macromolecule synthesis processes of the host cells. Indeed initiation in vitro of protein synthesis in rabbit reticulocyte lysates is inhibited by addition of poliovirus (Racevskis et al., 1976). Virus-associated kinases might phosphorylate cellular proteins after infection. In this context several host proteins must be considered as targets. The cap-binding protein with a molecular weight of 24000 (Sonnenberg et al., 1979) is inactivated after poliovirus infection (Trachsel et al., 1979). A protein of identical size is only phosphorylated in poliovirus-infected Vero cells and not in uninfected control cells (James & Tershak, 1981). Whether or not this phosphorylated protein is identical with the cap-binding protein is not yet known.

The activity of the initiation factor eIF2 is also regulated by phosphorylation and dephosphorylation (Ranu et al., 1978; Rosen & Krebs, 1981; De Haro et al., 1982). It will be of great interest to see whether poliovirus infection results in increased phosphorylation of eIF2 and of the cap-binding protein. Further studies are called for to elucidate the possible role of the poliovirus-associated protein kinase in the shut-off.

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