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Presence of Nucleoside Triphosphates and Calcium Associated with Mycobacteriophage I3

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Abstract. The association of nucleoside triphosphate molecules and calcium ions with purified particles of mycobacteriophage I3 has been documented. The content of nucleoside triphosphate has been determined to be 118 molecules per phage particle by equilibrium dialysis against labelled ATP or 148 molecules per phage particle by the direct determination of labelled nucleoside triphosphate. The concentration of bound Ca^{2+} exhibited a high degree of variation between different batches, which may be due to the nonspecific binding of Ca^{2+} by the virus particles. However, the tightly bound Ca^{2+} not removable by dialysis against calcium-specific chelating agent, showed a constant value of 2985 atoms/phage particle.

Key words: Bound NTPs – Injection of DNA – Phage bound Ca^{2+} – Equilibrium dialysis – Mycobacteriophage I3

Injection of DNA by mycobacteriophage I3 is accomplished with the aid of a contractile tail system and the process is inhibited by Tween-80 (polyoxyethylene sorbitan monooleate), a non-ionic detergent routinely used in mycobacterial cultures (Gadagkar and Gopinathan 1978). Calcium ions are necessary for the injection of DNA in minimal medium and for subsequent intracellular development of the phage (Nagaraja and Gopinathan 1980). In an earlier report (Karnik and Gopinathan 1980), we have shown the presence of a Ca^{2+} activated ATP hydrolysing activity associated with phage I3 which is inhibited by Tween-80. Calcium reverses the inhibitory effect of Tween-80 on the injection of DNA as well as on the ATPase activity. This suggests the possibility of involvement of ATPase activity in the injection of DNA, presumably during the contraction of the tail.

While the requirement for Ca^{2+} for successful phage infection is usually satisfied by supplementation of the ion to the growth medium, there is no tradition of providing ATP or other nucleoside triphosphates (NTPs) to the medium for successful phage propagation. Since the whole process of contraction takes place extracellularly the pool of NTPs of the host cell would not be available. Then where does the phage get the NTP molecules in the extracellular environment prior to infection? It is possible that the mature virus particles carry ATP (or other NTPs). In fact, the presence of nucleoside triphosphates has been reported in T-even phages (Wahl and Kozloff 1962).

In the present communication we describe the quantitative estimation of NTPs and Ca^{2+} associated with the mature phage I3 particles.

Materials and Methods

Bacteria and Phage. Mycobacterium smegmatis SN2 was obtained from R. Bonicke, Institute for Experimental Biology and Medicine, Borstel, Germany. Mycobacteriophage I3 was isolated by Sundarraj and Ramakrishnan (1970). The phage and bacteria were grown in a modified medium of Youmans and Karlson (1947) as described earlier (Karnik and Gopinathan 1980).

Purification of Phage. The phage was purified by a series of high and low speed centrifugations, followed by banding on a cushion of 60% (w/v) sucrose and then by centrifugation through a linear gradient of 15–60% (w/v) sucrose (Karnik and Gopinathan 1980).

Determination of Phage Bound NTPs. Approximately 3×10^{12} phage I3 particles labelled with ^{32}P (9×10^6 cpm in total) were extracted with ice-cold percholoric acid (10%). The perchloric acid soluble radioactivity was fractionated on Dowex-50 ion exchange resin (Cl^- form) along with carrier ATP, ADP and AMP. Elution was carried out using 0.1 N HCl essentially by the method of Katz and Comb (1963) as modified by Bhat (1978).

Equilibrium Dialysis. Equilibrium dialysis was carried out in microcells as described by Englund et al. (1969). The apparatus consisted of two compartments each of a capacity of 125 µl separated by a dialysis membrane. 125 µl of purified phage particles (2.5×10^{10} PFU) were taken in chamber I and varying concentrations of ligand in chamber II. The set up was kept at 4° C for 24–36 h to attain equilibrium. At the end of the period samples from each of the chambers were taken for determination of the concentration of the ligand. In another set of experiments the ligand and the phage were mixed in chamber I and dialysed against buffer in chamber II to attain equilibrium.

Estimation of Ca^{2+}. The content of Ca^{2+} in the purified phage was estimated by measuring the emission at 629 nm in an Elico flame photometer. A standard $CaCl_2$ solution (1 µg/ml) was used as reference. Phage preparations extensively dialysed either against buffer or 1 mM EGTA and water were directly used.

Results and Discussion

^{32}P labelled phage I3 lysates were prepared as described earlier (Karnik and Gopinathan 1980) in the synthetic medium where the only source of phosphate was ^{32}P orthophosphate (carrier-free, Bhabha Atomic Research Centre, Bombay, India). The presence of ATP was demonstrated by

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Abbreviations. EGTA, Ethylene glycol-bis (β-aminoethylether)-N,N¹ tetraacetic acid; PFU, plaque forming unit; NTP, nucleoside triphosphate

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isotope dilution method essentially as described by Kozloff and Lute (1959) for phage T2. The perchloric acid (10% v/v) extractable radioactivity constituted about 0.9% of the total phage radioactivity. The acid soluble extract was chromatographed on Dowex-50 after adding about 10 $A_{260\,nm}$ units of each of nonradioactive ATP, ADP and AMP as carriers. Nearly 37% of the acid soluble radioactivity eluted with carrier ATP. Under the conditions of our experiments no radioactivity corresponding to NDPs or NMPs (nucleoside di- or monophosphates) was eluted with carrier ADP and AMP. For quantitation of the NTP content, the values were normalised to the percentage recovery of the carrier ATP from the column. Total phage phosphorous was estimated by digesting the phage with 1N H_2SO_4 and estimating inorganic phosphorous in the digest by the method of Fiske and Subbarow (1925). By computing the ratio of radioactive phosphorous as NTP to total phosphorous per phage particle (6.68×10^{-18} g) and substituting these values in the formula given (Kozloff and Lute 1959), we arrived at the number of NTP molecules per phage particle.

$$\frac{\text{total NTP}}{\text{phage}} = \frac{^{32}\text{P(NTP)}}{\text{total phage } ^{32}\text{P}} \times \frac{\text{g P/phage}}{\text{P/ATP}} \times \text{molecules/mol.}$$

In two determinations, the number of NTP molecules per phage particle was found to be 145 and 150, giving an average value of 148. These values corresponded to 0.33% of total phage phosphorous. The chromatography on Dowex-50, however, does not distinguish between different ribo- or deoxyribonucleoside triphosphates.

If the NTPs were bound specifically to the phage it should be exchangeable with externally added NTP. In order to find this out, equilibrium dialysis was carried out by exchanging the bound ATP with increasing concentrations of ^{3}H-ATP (sp. act. 5.51 Ci/mmol, from BARC, Bombay, India) by the method of Englund et al. (1969). It is clear that ATP binding to phage particles can be saturated by exchange reaction. Saturation binding was obtained at concentrations above 40 pmol with 2.5×10^{10} phage particles in a volume of 125 µl (Fig. 1). Approaching the equilibrium from the other side, by mixing the ^{3}H-ATP with the phage and dialysing out the unbound ATP gave similar pattern of binding. The data given in Fig. 1 were analysed by Scatchard plot (Fig. 2) (Segel 1976). The pattern of binding appears to be straight-forward and does not show any interaction between the sites. The intercept on the abscissa gives the total bound ligand concentration, $n(\text{Po})_t$. Using this value and total number of phage particles $(\text{Po})_t$, the number of binding sites (n) per phage particle can be calculated as given below (Segel 1976):

$$n = \frac{\text{Mol of ligand bound} \times \text{Molecules/mol}}{(\text{Po})_t}$$

This value in our estimation was 118 ATP molecules/phage. The negative slope of the Scatchard plot (0.0654) gives directly, $-1/\text{Ks}$, from which the dissociation constant ($-\text{Ks}$) for the ATP-phage complex was obtained. The dissociation constant of 1.19×10^{-7} M thus obtained reflects fully the strength of binding.

This experiment does not rule out the displacement of other NTPs by ATP. Also, ATP at such sites which could not be exchanged are not distinguished. However, one can state that ATP binds with high specificity to phage particles in an exchange reaction. The discrepancy in the NTP bound per phage particle as measured by two different techniques might be due to the existence of NTPs other than ATP. For instance, the presence of dATP has been reported in phage T2 (Kozloff and Lute 1959; Wahl and Kozloff 1962). Studies on T2 (Wahl and Kozloff 1962) have revealed correlations between the

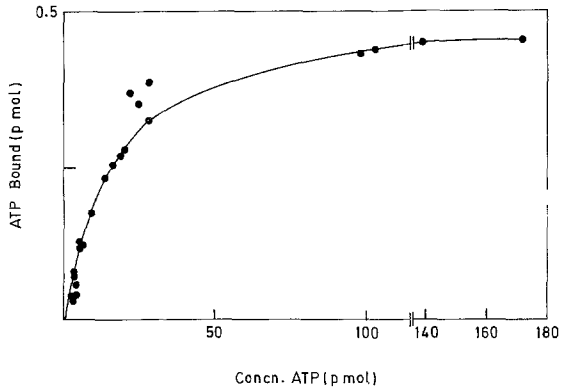

Fig. 1. Binding of ^{3}H-ATP to phage I3. 125 µl of purified phage (2.5×10^{10} PFU) was dialysed against varying concentrations of ^{3}H-ATP in a microcell to attain equilibrium at 4° C. At equilibrium, the radioactivity in both compartments were measured to obtain the respective bound and free values. The radioactivity bound to the phage in presence of a 1000-fold excess of unlabelled ATP was taken as nonspecific binding. Necessary corrections were made in the individual values to overcome nonspecific binding and also the ligand binding to dialysis membrane

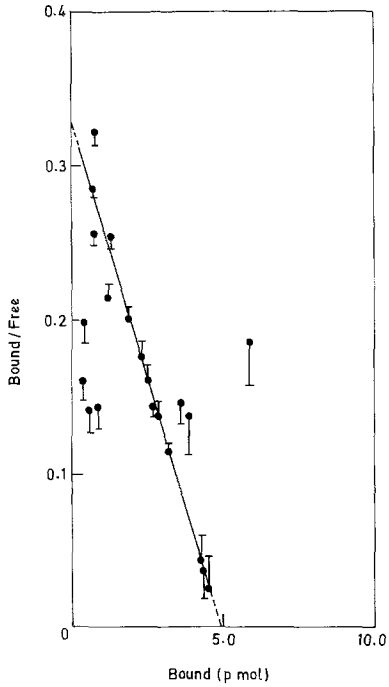

Fig. 2. Scatchard plot analysis of ATP binding. The binding data was analysed by Scatchard plot. The abscissa values are absolute amounts of ligand bound per 2.5×10^{10} phage particles at various concentrations of ligand in a volume of 125 µl. The individual points represent the average values ± SE, of two independent experiments done in triplicate. The slope of the plot was calculated by least-square fit analysis

Table 1. Calcium content of mycobacteriophage I3[a]

Preparation	Bound Ca^{2+} (Atoms per phage)
I	2,900
II	2,694
III	3,360

[a] 2×10^{12} PFU/ml of the purified phage were dialysed against 10 mM Tris-HCl pH 7.4 containing 1 mM EGTA and then against water with 3 changes. The final preparation was used for calcium estimation by flame photometry in an Elico flame photometer by measuring emission at 629 nm $CaCl_2$ (1 µg/ml) was used as standard. In all experiments the loss of phage viability was monitored separately and necessary corrections were made while calculating the Ca^{2+} per phage particle

total number of NTPs and the tail sheath subunit indicating a ratio of 1:1 on molar basis. However, such correlation in phage I3 is not possible as no information on the tail sheath subunits is available as yet.

To find out whether or not Ca^{2+} is associated with the mature phage particles the following experiments were done. The calcium content of the purified phage particles dialysed extensively against buffer was measured by flame photometry. The amount of Ca^{2+} associated with the phage showed a high degree of variation ranging from 12,000–36,000 atoms/phage. To check if this variation was due to the low affinity binding, the phage preparations were dialysed against 1 mM EGTA (a concentration that does not damage phage I3) and were used for the estimation of Ca^{2+}. In such cases the amount of Ca^{2+} bound to the phage showed a fairly constant value of 2985 atoms/phage on an average (Table 1). This value did not change when the EGTA concentration was increased during dialysis.

We have ruled out the possibility that the phage preparations used contain spurious calcium binding factors by re-equilibrating the EGTA dialysed phage preparation with ^{45}Ca and by gel filtration on Sepharose-2B column. The bound ^{45}Ca co-eluted with the viable phage particles (data not shown). Calculation of ^{45}Ca bound per phage particle again varied. This confirms the earlier observation that binding at sites accessible to EGTA chelation is highly variable.

When dialysed against EGTA it is possible that Ca^{2+} bound at high affinity binding sites might also be removed. Perhaps these sites are few in number and Ca^{2+} at such sites may have some role in the infection process. It should be possible to saturate such sites at lower ranges of concentration of ^{45}Ca at which low affinity binding sites would not be saturated. On equilibrium dialysis of EGTA dialysed phage, however, the binding of ^{45}Ca observed was highly erratic. Analysis of the data did not show any feature of the kinetics of high affinity binding (data not shown).

It would appear from our studies that there are two types of Ca^{2+} binding to the mature phage particles: (a) Binding at sites not removable by extensive dialysis against buffer but removed by EGTA; this must be due to simple association of Ca^{2+} with phage associated phospholipids. Phage I3 contains considerable amounts of phospholipids and free fatty acids amounting to about 16% of total mass of the phage particles (M.L. Gope and K. P. Gopinathan, manuscript submitted). These lipids appear to form a typical bilayer around the phage as seen in phage PM2 (Scharfer et al. 1974), and are essential for the integrity of phage structure and viability. Binding of Ca^{2+} might contribute to the stability of the lipid envelope. When dialysed against EGTA, there was reduction in the infectivity of the phage which could be restored by re-equilibration with Ca^{2+} (data not given). The stabilisation of membrane bilayer by calcium is well established in several cases (Carafoli and Crompton 1978). (b) The other class of binding sites are probably deeply buried and therefore are not accessible to chelation by EGTA. This part of the calcium appears to be integral part of the phage structure. Ca^{2+} associated in a similar fashion in phage T2, and not accessible to removal by citrate has been reported by Kozloff and Lute (1960). In addition, some of the calcium may also be associated with the DNA.

Since phage I3 contains large amounts of charged lipids interacting with the phage head, considerably large amount of Ca^{2+} bound may not be surprising. This Ca^{2+} might play a major role in the interaction of the membrane envelope and the phage proteins as Ca^{2+} has been shown in several cases to facilitate the association of proteins with membrane (Carafoli and Crompton 1978). Such interactions would play a major role in the structural integrity as well as in the assembly of the phage particle. In fact this might explain the absolute requirement for Ca^{2+} during the development of the phage I3 (Nagaraja and Gopinathan 1980).

Based on these and previously reported observations on T-even phages (Kozloff and Lute 1959; Wahl and Kozloff 1962), we propose that hydrolysis of bound NTPs is necessary for the injection of DNA in the mycobacteriophage I3. Interaction of the phage with the host receptor might induce a conformational change bringing the bound NTPs (which are otherwise inaccessible) to the site of ATP hydrolysing enzyme. The hydrolysis of NTPs leads to the contraction of the tail sheath and to the release of DNA.

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