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A permeabilized cell system that assembles filamentous bacteriophage

(membranes/ATP requirement/proton motive force)

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ABSTRACT **A permeabilized cell system has been developed that is capable of assembling filamentous phage only upon addition of exogenous thioredoxin. The *in vitro* system exhibits the same component requirements seen *in vivo*: functional thioredoxin, an intact packaging signal in the substrate DNA, and the assembly protein, pIV. This crude *in vitro* system is insensitive to inhibitors of protein or DNA synthesis, demonstrating that particle assembly uses components that had accumulated before cell permeabilization. The temporal separation of the synthetic period, during which phage proteins and DNA accumulate, from the assembly period enabled us to examine the energy requirement for assembly. We show here that ATP hydrolysis is required for filamentous phage assembly and that the proton motive force is also important.**

Filamentous phage particles are rods about 65 Å in diameter and 9,000 Å in length. They contain a circular single-stranded DNA (ssDNA) encapsulated in a tube formed by several thousand copies of the major coat protein. A base paired region of the ssDNA, the packaging signal (PS), is located at one end of the particle, as are four to five copies each of two minor coat proteins. The other end of the particle contains four to five copies each of two different minor coat proteins.

Upon entry into the cytoplasm, host enzymes convert the phage ssDNA to a super-coiled double-stranded form which is the template for rolling-circle replication and for phage gene expression. The five phage structural proteins are inner membrane proteins before their incorporation into the phage particle (1–3). Three phage-encoded proteins are required for assembly, but they are not part of the particle; pI and its internal restart, pXI, are in the inner membrane (4, 5), and the third, pIV, is in the outer membrane (6). In the cytoplasm, ssDNA-binding protein (pV) dimers coat the ssDNA, thereby collapsing it into a flexible rod. The PS remains exposed at one end of this complex (7), and genetic evidence suggests that it binds to pI at the membrane to initiate assembly (8). Assembly consists of the removal of pV from the ssDNA and its orderly replacement by the integral membrane coat proteins concomitant with particle extrusion. Extrusion across the bacterial membranes is a continuous process that does not rupture or kill the infected cell.

In addition to the phage proteins, assembly depends on host-encoded thioredoxin (9). In the absence of functional thioredoxin, coat protein accumulates in the membrane, but particle extrusion from the cell does not occur (10). It has been suggested that thioredoxin promotes the processive replacement of pV by coat proteins, probably by an interaction with the cytoplasmic domain of pI (2).

As with many other complex biological processes, filamentous phage assembly cannot be understood in detail without a suitable *in vitro* system in which the function of the relevant components can be determined. Hitherto, no satisfactory system had been developed. The membrane localization of both the protein components and the assembly apparatus suggests that a simple system would be hard to achieve. Even the removal of the outer membrane and peptidoglycan by conventional methods leaves the resulting spheroplasts unable to assembly phage (unpublished results).

We have established a system that represents a first step in the *in vitro* reconstitution of phage assembly. The system is based on the requirement for thioredoxin. By infecting cells that lack the thioredoxin gene, the necessary phage-encoded components accumulate, and virtually no particles are produced. When these thioredoxin-deficient, infected cells are permeabilized in the presence of purified thioredoxin, assembly takes place, and substantial numbers of particles are produced.

In vivo, synthesis of components and phage assembly occur at the same time. Hence it has not been possible to determine the requirements for assembly alone. This limitation has been overcome by separating (in time) component accumulation from assembly.

MATERIALS AND METHODS

Strains. *E. coli* K12 strain A307, a derivative of our standard filamentous phage f1 host strain, K38, in which the thioredoxin gene (*trxA*) has been deleted, was used for permeabilization. K1640, an A307 derivative in which the *unc* deletion, Δ*atp(IpIBEFHAGDC)*750 (11), was introduced by P1 transduction was used in some experiments. The recipient strain used to assay phagemid particles was K1018 (K38 *rep-71 ilvY*::Tn*10*). The *rep* mutation prevents f1 DNA replication, and the Tn*10* (transposon 10) specifies resistance to tetracycline (Tet^R).

The standard helper phage (R176) is an essentially wild-type f1 phage carrying all the phage genes. It carries an interference resistance (IR) mutation that renders it less susceptible to interference by a competing phage replication origin carried on a phagemid (12). R472 (ΔPS gene I^{sup} T96A IR) lacks a packaging signal and carries a suppressor mutation in gene *I* which enables it and phagemids that lack a packaging signal to be packaged (8). R184 (gene IV^{amber} IR) carries a chain-terminating mutation in gene *IV*.

The phagemid pMac 5–8 (13) was used to measure particle formation; this plasmid specifies chloramphenicol resistance

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Abbreviations: ssDNA, single-stranded DNA; PS, packaging signal; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; DNP, dinitrophenol; PMF, proton motive force; Cm^R, chloramphenicol resistance; Tet^R, tetracycline resistance; cfu, colony-forming unit.

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(Cm^R) and ampicillin resistance (Amp^R) and contains a ColE1 origin of replication, and the f1 origin of replication and packaging signal. Phagemids pPMR30 (Amp^R, ColE1 ori, f1 ori, f1 PS) and pPMR30 Δ17 (PS Δ17) were used where indicated. A307 cells containing plasmid pHH30 (14) or pPMR5-*trxA13* (15) were used to overproduce wild-type and mutant thioredoxin, respectively.

Chemicals. Wild-type and G92D mutant thioredoxin were purified as described (15). Natural nucleotides (ultrapure solution, 100 mM) were from Pharmacia; dideoxynucleotides (10 mM in 1 mM Tris·HCl, pH 7.2) were from BRL. AMP-PMP, GMP-PMP, and AMP-PCP (tetralithium) were from Boehringer Mannheim; ATPγS and GTPγS (tetralithium) were from Sigma. All were dissolved at a concentration of 100 mM in 10 mM Tris·HCl (pH 7.2). Stock solutions of 10 mM carbonylcyanide *m*-chlorophenylhydrazone (CCCP) in 100% ethanol and 500 mM dinitrophenol (DNP) in methanol were freshly diluted in water immediately before use.

Permeabilization Procedures. Cells were grown in fortified broth (6) at 37°C to $OD_{660} = 0.5$, infected by 8×10^9 helper phage per OD (≈20 phage per cell), and incubated for 1 h (final $OD_{660} \approx 1.0$).

Method A. The infected cultures were harvested, washed twice in an equal volume of ice-cold 30 mM Tris·HCl (pH 8.0), and suspended in one-tenth volume of the same buffer. Sucrose (20% final), EDTA (1 mM), and lysozyme (1 μg/ml) were added, giving one-fifth the original culture volume. After a 5-min incubation on ice, a 15 mM Tris·HCl, pH 8.0/20% sucrose solution was added to lower the EDTA and lysozyme concentrations 10-fold. After centrifugation and resuspension in the same 15 mM Tris·HCl/20% sucrose buffer, cells were divided into aliquots, each containing 0.4 OD_{660} cells (per reaction). After the final centrifugation and thorough removal of trace amounts of the sucrose buffer, each aliquot (0.4 OD_{660}) was suspended in 50 μl of reaction buffer A (10 mM Hepes, pH 7.5/20 mM $MgCl_2$/16 μM thioredoxin) with appropriate additional components where indicated and incubated at 25°C.

Method B. Cultures were washed twice in an equal volume of ice-cold 60 mM Tris·HCl (pH 7.1) and suspended in one-tenth volume of the same buffer. Sucrose (20% final) and EDTA (0.1 mM) were added, giving one-fifth the original culture volume. After a 10-min incubation at room temperature, cells were centrifuged, suspended in 30 mM Tris·HCl, pH 7.1/20% sucrose, and divided as described in Method A. Each aliqout (0.4 OD_{660}) of cells was then suspended in 40 μl of reaction buffer B (10 mM $MgCl_2$/16 μM thioredoxin), subjected to four freeze–thaw cycles (20 s in ethanol-dry ice, 1 min at 25°C), and incubated at 25°C.

Method C. Cells were washed twice in an equal volume of ice-cold 30 mM Tris·HCl (pH 7.5 or pH 8.0; or occasionally, 50 mM potassium phosphate, pH 7.5) and aliquoted (0.4 OD_{660} cells per reaction). Cells were then suspended in 20 μl of reaction buffer C (50 mM Hepes, pH 7.5/10 mM $MgCl_2$/16 μM thioredoxin), subjected to four freeze–thaw cycles as above, and incubated at 25°C.

RESULTS

Rationale/Experimental Design. A Δ*trxA Escherichia coli* strain was used as host to prevent phage assembly while synthesis of phage components was taking place. Synthesis of phage-encoded proteins and ssDNA appears to be normal in phage-infected thioredoxin mutants (10), but phage production is reduced by a factor of $\approx 10^8$ relative to $trxA^+$ strains (data not shown). Thioredoxin is small, soluble, and stable, and it can be purified to homogeneity in large quantities. Thus it seemed reasonable to introduce it into permeabilized cells. The essence of the experimental design is that the energy-requiring synthetic processes necessary to accumulate substrates for assembly, ssDNA, and phage proteins, will occur *in vivo*, and that assembly of particles would occur subsequently, after the introduction of thioredoxin.

Because we expected that the efficiency of phage assembly in this system would be very low, a sensitive biological detection system was desirable to monitor product formation. Although plaque formation is sensitive enough to detect a single phage, this assay could not be used here. Phage infection is necessary as the source of phage proteins and DNA, but significant numbers of residual input phage remain cell-associated even after extensive washing procedures. The background of plaque-forming input phage is too high for detection of low level phage production by a plaque assay. Instead, we used a system in which the input and output particles are different, such that only output particles are detected (Fig. 1).

Phagemids are autonomously replicating plasmids that encode antibiotic resistance and also contain the phage origin of replication and packaging signal but no phage genes (16). When infected with helper phage that provide all the phage-encoded proteins, phagemid-containing cells normally produce transducing particles, which consist of plasmid ssDNA encapsulated in a phage-like particle, and progeny helper phages. In cells lacking thioredoxin, phagemid (and phage) ssDNA accumulates after infection with a helper phage, but so few transducing or phage particles are produced that there is no background of transducing activity.

Transducing particles can be detected by their ability to confer antibiotic resistance to a recipient cell. The presence of a different selectable marker in the recipient cells allows them to be distinguished from the particle-producing (donor) cells. In these experiments, the phagemid confers Cm^R, and the

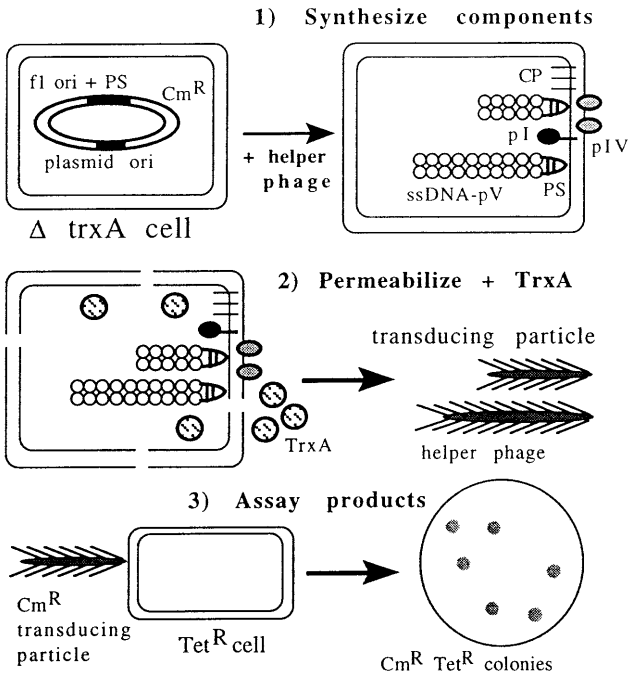

FIG. 1. Design of the system. (Step 1) Synthesis of components. Cells deleted for the thioredoxin gene (Δ*trxA*) and containing a phagemid conferring Cm^R were used. After infection by helper phage, the phage components necessary for assembly accumulate, but very few particles are made. (Step 2) Permeabilization. Cells were permeabilized in the presence of thioredoxin by one of three methods and incubated to allow particle production. (Step 3) Detection of product. An appropriate amount of the reaction supernatant was added to Tet^R recipient cells carrying the *rep* mutation (to prevent propagation of helper phage), and the mixture was spread on plates containing tetracycline and chloramphenicol. Transducing particles were scored as the number of Cm^R Tet^R colonies. The arrows indicate incubation periods.

recipients are Tet^R; hence, transducing particles are counted by adding them to recipient cells and scoring the number of Cm^R Tet^R colonies that arise. The recipient cells also contain a *rep* mutation, which prevents replication from phage origins (17) and thus prevents replication of helper phage but not the plasmid origin in phagemids (8). This scheme is outlined in Fig. 1.

Cell Permeabilization/Disruption. A variety of cell disruption methods were tested. In general, those that were most disruptive were least efficient in assembling particles. In particular, spheroplasts and spheroplast fragments failed, as did right-side-out vesicles (18) and translocation-competent inverted inner membrane vesicles (19).

We therefore developed gentler permeabilization procedures (Table 1) that differed in the degree of cell disruption they caused. In the first, sucrose was present to stabilize cells during lysozyme treatment, and the amount of lysozyme added was much lower than is usual (Method A). Under such conditions, transducing particle production was detectable after thioredoxin addition, although at a low level [10^5 colony-forming units (cfu) per OD_{660} permeabilized cells]. A freeze–thaw step in the absence of lysozyme but after plasmolysis with sucrose (Method B) gave a 100-fold higher yield of product. A simple freeze–thaw treatment (Method C) increased the yield an additional 10-fold. No particles were detected when thioredoxin was added to untreated cells or when the permeabilized cells were kept on ice.

For each permeabilization method, the addition of thioredoxin during permeabilization gave higher product yields than when it was added 10 min later (Table 1). Furthermore, when cells were permeabilized by Method B in the presence of thioredoxin, washed, and then resuspended in the absence of thioredoxin, the particle yield was 55% of that obtained when thioredoxin was present in the resuspension buffer. These results suggest that the permeabilized state is transient, and that the cells or vesicles reseal rapidly after dilution from sucrose and/or freeze–thaw.

The yield of particles was strongly dependent on the amount of thioredoxin added and saturated at about 10 μM (Fig. 2). Holmgren *et al.* (20) have determined that thioredoxin is present at about 12,000 molecules per cell; using 3.9×10^{-16} liters as the volume of an *E. coli* K12 cell, the normal thioredoxin concentration in *E. coli* is 50 μM.

Particle formation starts very quickly after the addition of thioredoxin; extrapolation of the rate suggests that the first transducing particles are formed within 1–2 min. Particle formation is essentially linear for 40–70 min (Fig. 3). In most subsequent experiments, thioredoxin was added at 16 μM, and the reactions were incubated for 80–90 min.

Assembly in Permeabilized Cells Reflects *in Vivo* Requirements. Phage assembly *in vivo* requires, in addition to the virion structural proteins, a cis-active element in the ssDNA (the PS) (16), two phage-encoded accessory proteins (pI and pIV), and host-encoded thioredoxin (1). To determine whether these requirements are maintained in the permeabilized cell system, purified mutant thioredoxin (TrxA G92D) that retains redox activity but fails to support phage assembly *in vivo* (15) was added. Mutant thioredoxin did not support product formation (Table 2). A helper phage carrying a gene *IV* nonsense mutation was used to assess the requirement for pIV in the permeabilized cell system; almost no transducing particles were assembled in the absence of pIV. The residual level of particles is due to am^+ revertants in the helper phage stock. Phagemids contain both a filamentous phage origin of DNA replication and a packaging signal. A modified phagemid in which the packaging signal had been deleted assembled particles at 0.3% of the control value. Deletion of the packaging signal does not affect the level of phagemid ssDNA synthesized *in vivo* (21).

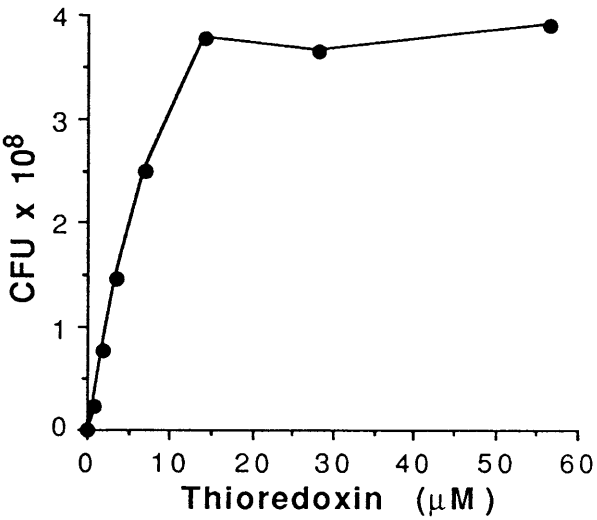

FIG. 2. Titration of thioredoxin for phage assembly. Cells were treated by Method C in the presence of thioredoxin at the indicated concentrations. The total protein concentration was kept constant at 160 μg/ml with BSA. The incubation time was 100 min.

Suppressor mutants that enable phage lacking a packaging signal to assemble *in vivo*, albeit less efficiently than wild-type phage, have been isolated (8). Helper phage containing such a suppressor mutation in gene *I* (R472) supported production of ΔPS phagemid particles at 25% of the level of PS-containing phagemids (Table 2), similar to the *in vivo* results. Thus with regard to these tests, assembly in the permeabilized cell system mimics the *in vivo* situation.

Particle Assembly by Permeabilized Cells Does Not Require *de Novo* RNA, Protein, or DNA Synthesis. To determine whether particle assembly was independent of concurrent RNA or protein synthesis, rifampicin or the protein synthesis inhibitor tetracycline was included with thioredoxin during permeabilization. Particle production showed similar kinetics

Table 1. Comparison of cell disruption methods

Method	Treatment*	cfu†	Relative yield:‡ TrxA at 10 min vs. TrxA at 0 min
A	Sucrose-EDTA-lysozyme	$1.3 \pm 0.7 \times 10^5$	0.2
B	Sucrose-EDTA; freeze–thaw	$2.2 \pm 1.3 \times 10^7$	10^{-4}
C	Freeze–thaw	$1.7 \pm 1.6 \times 10^8$	10^{-3}
	No treatment	$<10^2$	ND

TrxA, thioredoxin; ND, not done.
*Details are given in *Materials and Methods.*
†Average of four to five experiments.
‡Samples were either resuspended in thioredoxin-containing buffer (0 min) or resuspended without thioredoxin and kept on ice for 10 min before thioredoxin addition. The samples were then incubated at 25°C for 100 min.

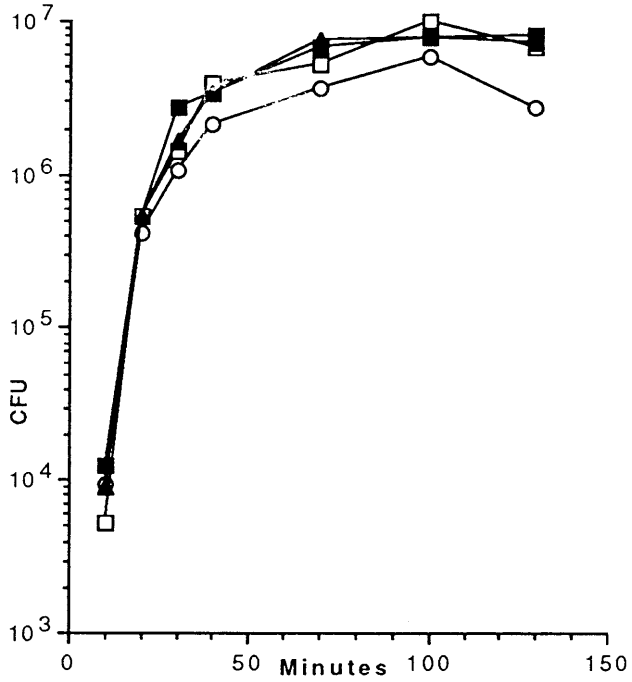

FIG. 3. The effect of inhibitors on particle assembly. Cells were treated with Method B. Inhibitors were added at the same time as thioredoxin. ■, No addition; ○, 20 μg/ml tetracycline; □, 100 μg/ml rifampicin; ▲, 1 mM dideoxycytidine and 1 mM dideoxythymidine triphosphate.

at early times, independent of whether inhibitors were present; more than 10^6 particles were detected by 50 min in both cases (Fig. 3). Tetracycline added *in vivo* to *trxA*$^+$ cells at the time of infection with helper phage completely blocked subsequent transducing particle production (data not shown).

A control system was devised to confirm that tetracycline was effective in blocking protein synthesis during the assembly phase of the reaction in permeabilized cells. Helper phage and thioredoxin were added at the time of permeabilization of uninfected cells. Under these conditions, prior accumulation of phage proteins has not occurred, and particle production relies entirely on proteins synthesized after permeabilization. Under these conditions, a low number of particles (4.5×10^4) were produced after 100 min of incubation (data not shown). When tetracycline was added at the same time as the helper phage and thioredoxin, no assembled particles were found (50 could have been detected). Similarly, 1 mM dideoxythymidine and dideoxycytidine triphosphates, added to block DNA synthesis, did not affect the yield of particles when added after components had been accumulated (Fig. 3) but completely abolished assembly in the control system (data not shown). Thus in the system that allows accumulation of phage precursors, particle assembly is independent of *de novo* RNA, protein, and DNA synthesis; more than 10^8 particles (under Method C) can be produced from presynthesized assembly components.

Table 2. Requirements for particle assembly

Experiment	Defective component	Relative yield
a	Thioredoxin (G92D)	0.0000
b	pIV	0.0001
c	PS	0.003
d	PS (pI suppressor of ΔPS)	0.25

Experiments: a, wild-type thioredoxin vs. mutant thioredoxin containing a Gly-92 → Asp substitution; b, R172 (standard helper phage) vs. R184 (gene *IV*^{amber} helper phage) and pMac5-8 (standard phagemid); c, pPMR30 (wild type) vs. its packaging signal deletion derivative, pPMR30Δ17 (ΔPS). These phagemids confer Amp^R; therefore, transducing particles were scored as Amp^R Tet^R colonies. d, helper phage R472, containing a gene *I* suppressor of the ΔPS mutation and phagemid pPMR30 vs. pPMR30Δ17. Experiments were by Method B with incubations of 130 min, except for b, which was by Method C with an incubation of 100 min.

Phage Assembly Requires ATP Hydrolysis. Arsenic acid inhibits ATP synthesis and markedly lowers cellular ATP levels (22). This inhibitor drastically reduces particle assembly in our system (Fig. 4). The reduction of ATP levels reduces the proton motive force (PMF) through the action of the F_1F_0-ATPase, which uses PMF to regenerate ATP when the cellular level is low (23). To uncouple the interconversion of ATP and PMF, we also examined the effect of arsenic acid in permeabilized cells in which the *unc* genes encoding the F_1F_0-ATPase had been deleted (11). The results (Fig. 4) indicate that low ATP levels directly affect particle assembly. Addition of ATP or GTP along with arsenic acid partially reversed the inhibition (Fig. 4).

Low ATP levels can affect many other metabolic processes, and in particular the level of other nucleotides. Furthermore, arsenic acid could also act as a competitive inhibitor of an NTPase required for assembly. To obtain more direct evidence for a specific nucleotide requirement, nucleotides or nucleotide analogs were added to the reactions (Fig. 5). Assembly is quite effective in the absence of any added nucleotides and is stimulated by the addition of many of the compounds tested, including all of the natural nucleotides, P_i, and even some analogs. This stimulatory effect could be due to increased ATP levels resulting from synthesis, interconversion of natural nucleotides, or inhibition of non-assembly-related ATPases by the analogs. In strong contrast, the nonhydrolyzable ATP analogs AMP-PNP and AMP-PCP reduce the efficiency of particle formation to 3% of the control value. GMP-PNP does not inhibit. Another type of analog (ATPγS), which can be hydrolyzed by some but not all ATPases (24–27) does not inhibit. Product analogs (AMP + PP_i, GMP + PP_i, and TMP + PP_i) were also tested; only AMP + PP_i inhibited the reaction significantly (4% of control). Nucleoside monophosphates alone had no effect, and PP_i alone had a modest inhibitory

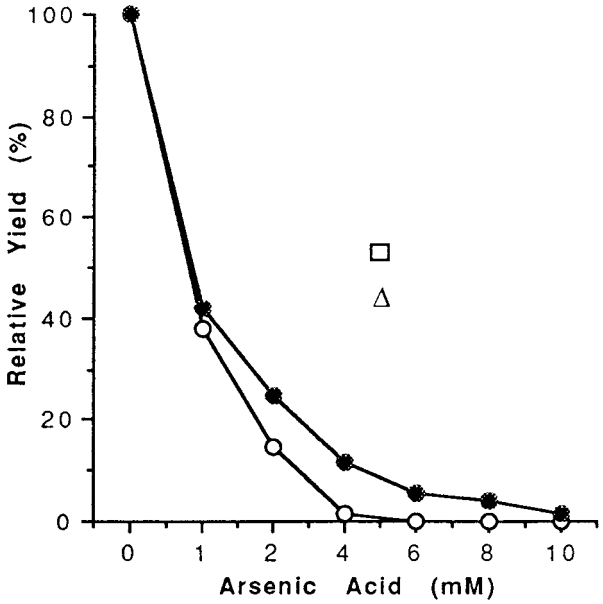

FIG. 4. Inhibition by arsenic acid and reversal by ATP and GTP. Cells were treated by Method A with an incubation of 80 min. All compounds were added at the same time as thioredoxin. Tetracycline (20 μg/ml) was added to prevent residual protein synthesis. ●, A307 (*unc*$^+$); ○, K1640 (Δ*unc*); □, K1640, 5 mM arsenic acid plus 10 mM ATP; △, K1640, 5mM arsenic acid plus 10 mM GTP.

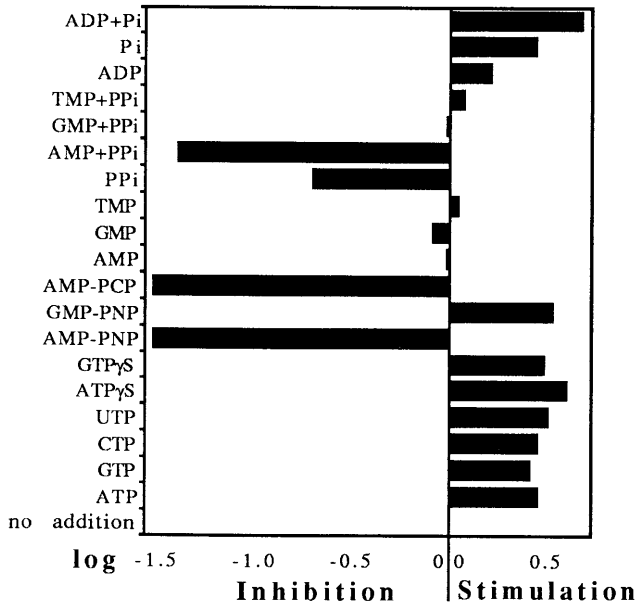

FIG. 5. The effect of nucleotides and nucleotide analogs. K1640 (Δ*unc*) cells were treated by Method A followed by an incubation of 80 min. All compounds were added at the same time as thioredoxin and were present at 10 mM (except for PP_i at 5 mM). Tetracycline (20 μg/ml) was added to prevent residual protein synthesis.

effect (20% of control). The only specific and pronounced effects on assembly were obtained when ATP analogs or hydrolysis products were used. A Δ*unc* strain was used for the study presented here; however, similar results were obtained with an *unc*$^+$ strain. Taken together, these results indicate that ATP hydrolysis is required for filamentous phage assembly.

PMF Dissipaters Inhibit Phage Assembly. Two different PMF dissipaters, CCCP and DNP, were used to examine the role of PMF in particle assembly. Both reagents effectively inhibited assembly (Fig. 6). In the *unc*$^+$ strain, CCCP (1 μM) reduced the reaction to 0.06% of the control, and DNP (300 μM) reduced the reaction to 0.2%. The Δ*unc* strain, which lacks the primary ATP/PMF interconversion pathway (23), was equally sensitive to these dissipaters. This initial result suggested that PMF is required for particle assembly, providing there is no conversion in the mutant strain. However, when ATP (10 mM) was included in the reaction in addition to CCCP (1 μM), the reaction efficiency in the mutant strain recovered to 40% of the no addition control, more than a 100-fold increase compared with CCCP alone (Table 3). The *unc*$^+$ strain gave only a 2-fold increase. This indicates that, contrary to expectation, the ATP level is affected by PMF even in the absence of the F_1F_0-ATPase. The reversal of CCCP inhibition is at least partially due to the direct effect of increased ATP concentration in the Δ*unc* strain. Even here, further increases in the CCCP concentration make the reaction very inefficient despite the presence of 30 mM ATP (Table 3). This suggests a direct requirement for PMF.

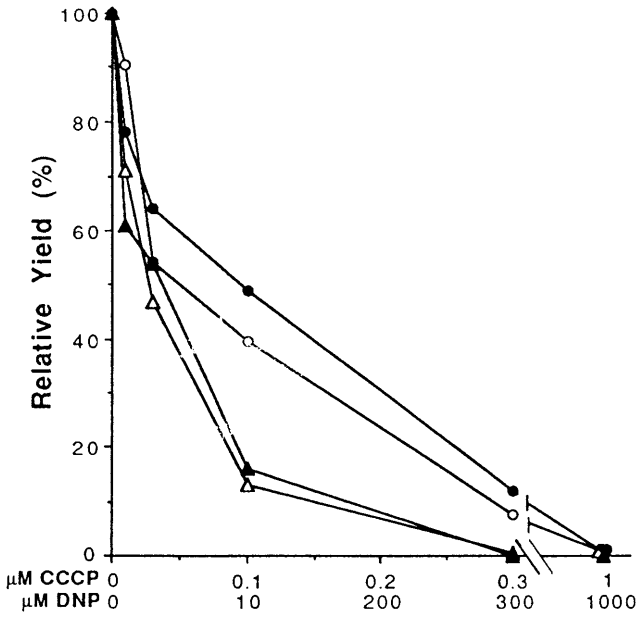

FIG. 6. Effect of CCCP and DNP. Cells were treated by Method A followed by an incubation of 80 min. CCCP and DNP were added at the same time as thioredoxin. Tetracycline (20 μg/ml) was added to prevent residual protein synthesis. ●, A307 (*unc*$^+$), CCCP; ○, K1640 (Δ*unc*), CCCP; ▲, A307 (*unc*$^+$), DNP; △, K1640 (Δ*unc*), DNP.

DISCUSSION

We have established a crude *in vitro* system that mimics the *in vivo* requirements for filamentous phage assembly and allows us to study the process in more detail than is possible *in vivo*. At its best, the permeabilized system assembles about 1 particle per cell in 1 h. Although this represents only about 0.1–1% of the *in vivo* rate, it is more efficient than many other *in vitro* virus assembly systems. We have not yet succeeded in getting the extracts to incorporate exogenously added DNA, and thus the product detected probably reflects the completion of particles whose assembly had been initiated *in vivo*. Nonetheless, it has been possible to temporally separate the accumulation of assembly components (coat proteins, DNA substrate, and assembly apparatus) from the assembly process, and this has enabled us to study the energy requirements for particle formation.

A central question in the study of any macromolecular transport/assembly system is what type of energy is used. Phage production requires metabolically active cells and is sensitive to cyanide or the removal of nutrients (1). Ng and Dunker (28) attempted to determine the energy requirement for filamentous phage assembly on the basis of *in vivo* experiments but could not really separate effects on assembly from those on protein synthesis.

In the assembly reaction, all nucleotides that contain a pyrophosphate linkage stimulated, except for AMP-PNP and AMP-PCP, which inhibited strongly. Since this is a relatively crude system, the simplest interpretation of the stimulation by nucleotides other than ATP is that they increase ATP levels, either by internucleotide conversion or because they inhibit or serve as alternate substrates for ATPases. ATPγS (and GTPγS) is also stimulatory; although this nucleotide is often an inhibitor of ATP-utilizing reactions, some enzymes (especially

Table 3. Reversal of CCCP inhibition by nucleotides

Additions	Relative yield, %	
	unc$^+$	Δ*unc*
None	100	100
1 μM CCCP	0.2	0.3
1 μM CCCP + 10 mM ATP	0.5	40
3 μM CCCP + 10 mM ATP	0.4	30
10 μM CCCP + 10 mM ATP	0.04	12
30 μM CCCP + 10 mM ATP	<0.02	5
30 μM CCCP + 30 mM ATP*	ND	1.3
30 μM CCCP + 10 mM AMP/GMP-PMP	<0.02	<0.04

Experimental conditions were as in Fig. 6. ND, not done.
*From a separate experiment in which Mg^{2+} was 40 mM to compensate for the higher ATP. The control experiment (40 mM Mg^{2+} + 30 mM ATP without CCCP) had similar value as the one done at the standard condition (20 mM Mg^{2+} + 10 mM ATP).

kinases) can readily utilize it (25), probably because the sulfur is not in a bridging position. AMP-PNP, AMP-PCP, and AMP + PP_i were potent inhibitors, whereas GMP-PNP, TMP + PP_i, and GMP + PP_i were not. These results show that ATP hydrolysis is required for f1 assembly.

Filamentous phage pI contains a well conserved Walker nucleotide binding motif (2), and a single amino acid change in the motif abolishes pI function (unpublished work). Hence it seems reasonable to propose that pI hydrolyzes ATP to catalyze assembly. Assembly is sensitive to PMF dissipaters even when the PMF is largely uncoupled from ATP metabolism in a mutant strain deleted for its F_1F_0-ATPase. Hence in addition to a requirement for ATP hydrolysis, a membrane potential is needed for assembly, as is the case for protein translocation (29). The requirement for a PMF also means that in our preparations, assembly is probably taking place in closed compartments. This is consistent with our observations that the more disruptive the treatment, the lower the yield of particles, and that assembly is more efficient when thioredoxin is added at the time of disruption. The vesicles likely reseal rapidly because the thioredoxin could be removed after permeabilization but before assembly started, with only a 45% loss in efficiency. Furthermore, the more disrupted the cells, the more assembly tolerates the late addition of thioredoxin. It may be that more disrupted vesicles are slower to reseal. A secondary implication of the PMF requirement is that *in vitro* assembly will require a closed system.

This study also shows that thioredoxin participates in assembly directly. The thioredoxin requirement is saturable at a concentration well below that calculated to be present in the cell, and therefore, thioredoxin probably acts in concert with another assembly component that is limiting. Since a suppressor for a thioredoxin missense mutation has been identified in gene *I*, the most likely candidate for this other component is pI.

Lysozyme cleaves the petptidoglycan but also leads to loss of the periplasmic contents and much of the outer membrane (18). From its strongly inhibitory effect on *in vitro* assembly, and from experiments that suggest that phage cannot be assembled in spheroplasts (J. L. Brissette, J.-n.F., and B. I. Kazmierczak, unpublished work), it seems likely that the peptidoglycan and outer membrane are directly or indirectly required for an effective assembly site. This inference, together with the PMF requirement, suggests that a truly soluble system will be difficult to achieve.

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