The Tail Fibre of Bacteriophage T4 is Sensitive to Proteases at Elevated Temperatures

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Bacteriophage T4 wild-type is not sensitive to heating at 60 °C. Trypsin at this temperature quickly inactivates the bacteriophage, with first-order kinetics for about the first 60 min. The half-inactivation period is around 15 min. The characteristics of this inactivation reaction have been studied. Inactivation of T4 particles is paralleled by a loss in ability to adsorb to bacteria. SDS-PAGE reveals a 'clipping' of gp 37, the protein of the distal part of the long tail fibres, during the inactivation reaction. gp 37 is the only protein to be modified under these conditions. Mutants have been isolated which resist this modification; they map in gene 37.

INTRODUCTION

Nucleic acids of all viruses are protected against the environment during the vegetative period of their cycle by shielding surface structures. *Escherichia coli* bacteriophages, such as T4, are surprisingly resistant to attack by environmental factors, whether physical, chemical or biochemical (for example, proteases) (To *et al.*, 1969). They are much more resistant than the bacteria that support their growth. Hence, it seemed interesting to study the resistance of phage T4 to trypsin for two reasons: to gather data on the fragility/resistance phenomenon, and as a basis for understanding the role of proteases in the life cycle of the virus. The idea that *in vitro* proteolysis mimics *in vivo* proteolysis is well documented (Goldberg, 1972; Goldberg & Dice, 1974; Goldberg & St John, 1976). T4 wild-type particles are resistant to trypsin; they become sensitive to trypsin only when heated above 60 °C. I shall describe this inactivation reaction, and also the isolation and properties of a resistant mutant that maps in gene 37, the gene coding for the protein (designated gp 37) of the distal part of the long tail fibres.

METHODS

Genetic designations. *am*, amber; *ptr*, resistant to inactivation by trypsin at 60 °C; *ts*, thermosensitive; *wt*, wild-type; *gp*, gene product.

Chemicals. Enzymes: trypsin (Serva) from bovine pancreas, TPCK (L-1-tosylamide-2-phenylethyl chloromethyl ketone) treated, 40 U ml⁻¹ (trypsin crude extract, P. H. Stehelin, Basel, Switzerland, was used for screening experiments); α -chymotrypsin from bovine pancreas, type II, Sigma; trypsin soya bean inhibitor no. 24020, Merck; pronase, lyophilized, B grade, Calbiochem; deoxyribonuclease, crude extract, Sigma. Other chemicals: lactose, Siegfried, Zofingen; sucrose, ultrapure, Schwartz/Mann; [³⁵S]methionine was a gift of K. Suda; [³H]thymidine was obtained from Amersham.

General techniques. The methods used for bacterial and phage culture, sucrose gradient centrifugation, SDS-PAGE and autoradiography, and electron microscopy have been described (Adams, 1959; Showe *et al.*, 1976).

Proteolysis with trypsin: standard conditions. Phage stocks at concentrations from 10¹² to 10⁸ phage ml⁻¹ were diluted 1 to 10 in phosphate buffer in the presence of Mg²⁺ (10⁻³ M) (Showe *et al.*, 1976) and trypsin (100 µg ml⁻¹). The trypsin stock solution was kept at 4 °C at 1000 µg ml⁻¹ in 10⁻³ M-HCl solution: activity was preserved for several weeks. For inactivation, the suspension was heated to 60 °C. At various times the suspension was agitated for about 10 s and a sample (50 to 100 µl) was removed and cooled to 20 °C.

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Table 1. *Bacterial and phage strains*

<i>E. coli</i> strains*		Relevant properties		
B ^E		Non-permissive for amber		
CR63		Permissive for amber		
K12(λ)		Non-permissive for r ^{II}		
B/4		Resistant to T4		
Phage T4 mutants used in crosses to locate the <i>ptr</i> mutant*		Other phage T4 mutants		
Gene	Mutant	Gene	Mutant	Source†
r ^{II}	r 219	23	<i>am</i> H 11	1
5	<i>am</i> B 256	23	<i>am</i> H 32	1
10	<i>am</i> B 255	23	<i>ts</i> H 86	1
23	<i>am</i> H 32	36	<i>am</i> E 1	1
27	<i>am</i> N 120	37	<i>am</i> N 52	1
57	<i>am</i> E 198	37	<i>am</i> B 34	2
31	<i>am</i> N 54	37	<i>am</i> B 280	1
33	<i>am</i> N 134	37	<i>am</i> NG 187	3
37	<i>am</i> B 280	37	<i>am</i> NG 475	3
43	<i>am</i> B 22	38	<i>am</i> B 262	1
44	<i>am</i> N 82	38	<i>am</i> A 470	2
49	<i>am</i> E 272	38	<i>am</i> H 41	3

* All from this laboratory.

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Selection of ts revertants of am mutants: screening of single plaques for trypsin resistance. A typical experiment is described. Double mutants *ptr-am x* (*x* stands for the mutant designation) (Table 1) were plated at 10^8 per plate on *E. coli* B^E and incubated at 32 °C overnight. Single plaques were checked for their *ts* character. Samples of the *ts* plaques were suspended in 1 ml of phosphate buffer containing trypsin ($100 \mu\text{g ml}^{-1}$) and treated with chloroform. A drop of this suspension was laid on a lawn of bacteria; the suspension was further incubated at 60 °C for 1 h and then spot tested again. Comparison of the lysis before and after incubation allowed resistant plaques to be detected. Plaques were purified, and lysates were made from purified plaques and checked for trypsin resistance.

Complementation. Exponentially growing bacteria in medium M9 AA (Showe *et al.*, 1976) at 2×10^8 bacteria ml^{-1} were infected and 9 min later superinfected to a total m.o.i. of 10. After 1 h, the culture was lysed with chloroform. The input ratios and non-permissive conditions (strain and temperature) are described for each experiment. All crosses were made under the same conditions.

Radioactive labelling. Fresh overnight cultures were diluted 1 to 100 in M9 medium and grown to a concentration of $2 \times 10^8 \text{ ml}^{-1}$. Labelling was done in a second subculture in M9 medium supplemented with 10^{-4} M-MgSO₄ for methionine labelling, or with $10^{-2}\%$ Casamino acids (Difco) for thymidine labelling. Timing and concentrations are indicated for each experiment.

RESULTS

Effect of trypsin and temperature on T4 wild-type phage infectivity

Bacteriophage T4 wild-type is not sensitive to heating at 60 °C (Arscott & Goldberg, 1969; Kao & McClain, 1980) or to trypsin below this temperature. However, it was rapidly inactivated in the presence of trypsin at 60 °C. The inactivation followed first-order kinetics (Fig. 1) with a first-order rate constant calculated for 30 different single plaques after 30 min incubation of $k = 0.172 \text{ min}^{-1}$ (s.d. 0.03 min^{-1}). Without trypsin, there was a slow inactivation, with some variation from experiment to experiment, with k about 0.003 min^{-1} . Besides the fluctuation of the response of individual plaques to trypsin treatment, there seemed to be some variation of susceptibility from stock to stock.

During inactivation the morphology of the plaques became heterogeneous and more and more plaques were minute. First-order kinetics stopped after about 1 h; about one phage in 10^4 remained viable. Curves were of the same type at pH 5 (at this pH long tail fibres are in the up position: Kellenberger *et al.*, 1965). Inactivation curves were the same for sucrose-purified stocks as for crude lysates.

Fig. 1. Trypsin inactivation of phage. Trypsin inactivation was carried out both under the standard conditions and after addition of soya bean trypsin inhibitor [$1.2 \text{ mg (mg enzyme)}^{-1}$] at 0, 5, 15 and 30 min after the beginning of inactivation (arrows). The figure is a semi-logarithmic plot of the surviving fraction as a function of time. ●, Wild-type; ○, *ptr* mutant.

Selection of a resistant mutant

It seemed interesting to isolate a mutant resistant to this inactivation, and to investigate its phenotype and its genetic localization. Such a phage could not be obtained by single-step selection: several successive cycles of proteolysis were required. I propose to call this variant *ptr*.

Escherichia coli was grown in M9 AA medium (Showe *et al.*, 1976) to a concentration of 2×10^8 bacteria ml^{-1} . After low-speed centrifugation, the bacteria were resuspended at $2 \times 10^9 \text{ ml}^{-1}$ in phosphate buffer and aerated for 15 min. Chymotrypsin-treated phage were adsorbed to these bacteria at an m.o.i. of about 0.01 or 0.1, diluted 1 to 10 in M9 AA and aerated for 2 h. The chloroform-treated lysate was treated in the same way and submitted to another cycle of growth. After seven cycles, the fraction of survivors was 40% and *ptr* mutants were easily found by screening isolated plaques for resistance. Similar selections were performed on T4 strain 23 (*am* H 32) grown on *E. coli* CR63 at 37°C and on T4 strain 23 (*ts* H 86) grown on *E. coli* B^E at 32°C . *am*⁺ and *ts*⁺ derivatives were subsequently isolated by crossing. In all experiments, the behaviour of these two mutants was the same.

The *ptr* mutants resist trypsin inactivation: they show a slow inactivation, exponential for at least 2 h with $k = 10^{-3} \text{ min}^{-1}$ (Fig. 1). This rate of inactivation was about the same as that of the wild-type without trypsin at 60°C , but it seemed more reproducible: inactivation of a *ptr* mutant seemed a better reference for a definition of wild-type inactivation than the spontaneous inactivation without trypsin of the wild-type. The titre of *ptr* strains sometimes increased at the beginning of the inactivation. This effect was found with crude lysates.

Characterization of the inactivation reaction: comparison between the wild-type and a *ptr* mutant

To investigate the reliability and the accuracy of the test, the same isolated single plaque was counted twice. Fifty single plaques were collected at random from the offspring of coinfection by wild-type and a *ptr* mutant (at an m.o.i. of 5 for each phage). For each single plaque, counting was performed after two different periods of inactivation, 30 min and 60 min. In the regression graph of $k_{60 \text{ min}}$ versus $k_{30 \text{ min}}$ (not shown), the points were grouped in two clusters: 21 in the resistant zone, 29 in the sensitive zone. In spite of the highly significant difference between the two clusters (sensitive and resistant) the reproducibility inside each cluster was poor (correlation coefficient $r = 0.136$ for the sensitive and $r = 0.028$ for the resistant).

Table 2. *Comparison between trypsin and pronase inactivation of phages*

Inactivation was carried out under the standard conditions ($100 \mu\text{g trypsin ml}^{-1}$) at 60°C and compared with pronase inactivation ($10 \mu\text{g ml}^{-1}$): no significant difference was observed.

Phage	Protease	Fraction of phage surviving after incubation time (min) shown:						
		10	20	30	45	60	90	120
<i>ptr</i>	Trypsin	1.5	0.8	0.6	0.6	0.5	0.8	0.7
	Pronase	0.7	0.7	0.7	0.5	0.5	0.2	0.5
wt	Trypsin	0.3	0.06	0.01	9×10^{-4}	3×10^{-4}	7×10^{-5}	9×10^{-5}
	Pronase	—	0.08	0.01	8×10^{-4}	3×10^{-4}	3×10^{-5}	6×10^{-5}

Table 3. *Reversibility of heat treatment of phage*

Several phage stocks were treated for 1 h at 60°C with and without trypsin, and at 37°C with trypsin. Phages exposed to high temperature recovered their infectivity after cooling.

Treatment	Fraction of phage surviving after treatment				
	<i>ptr</i> (sucrose-purified)	wt (fresh lysate)	wt (old lysate)	wt (sucrose-purified)	37 (<i>am B 280</i>) (fresh lysate)
37°C with trypsin	0.84	1.22	0.91	0.79	1.09
60°C without trypsin	0.51	0.51	0.37	0.41	0.27
60°C with trypsin	0.60	4×10^{-4}	6×10^{-5}	10^{-4}	3×10^{-4}

Table 4. *Effect of temperature on phage inactivation*

Samples of a wt stock were incubated with trypsin ($100 \mu\text{g ml}^{-1}$) at different temperatures. The first-order rate constants (k) are given.

Temp. . .	37°C	47°C	55°C	60°C	65°C	75°C	80°C
k_{initial} (min^{-1})	$< 10^{-4}$	$< 10^{-4}$	10^{-3}	0.16	0.70	1.00	1.60
$k_{90 \text{ min}}$ (min^{-1})	2×10^{-4}	8×10^{-4}	10^{-2}	0.95	0.12	0.12	0.13

The reaction was not specific for the protease used: inactivation curves for wild-type phage were the same for trypsin as for pronase, i.e. for a highly specific enzyme and for an enzyme of low specificity. Conversely, a *ptr* mutant was resistant to both enzymes (Table 2).

Inactivation was not stopped by dilution of the reaction mixture at 60°C (data not shown). Lowering the temperature to room temperature stopped the inactivation as testified by the inactivation curves. Another way to stop the reaction was the use of the soya bean trypsin inhibitor: addition of 1.2 mg of inhibitor per mg of enzyme stopped the inactivation immediately (Fig. 1). Trypsin inhibitor was always used when proteins had to be analysed by SDS-PAGE.

There was a strong transition effect at 60°C : below this temperature, practically no inactivation was observed. Since heating without enzyme did not inactivate the virus, inactivation works in two steps: the first a reversible transition necessary for the second, the irreversible inactivation by trypsin (Table 3). When the temperature of the reaction was increased progressively to 80°C , the initial velocity of inactivation increased; but the final extent of inactivation was not very much changed, as shown by the values of k at 90 min (Table 4).

Maximum inactivation was obtained with 100 or $1000 \mu\text{g trypsin ml}^{-1}$. At lower concentrations, the efficiency decreased very quickly, and $1 \mu\text{g ml}^{-1}$ was practically without effect (data not shown). A standard concentration of $100 \mu\text{g ml}^{-1}$ was used. Thermal

Fig. 2. Kinetics of loss of adsorbability of phage particles during trypsin treatment. After trypsin treatment for 0, 5, 20, 30 and 60 min, [^3H]thymidine-labelled r^{II} phage were adsorbed at 37 °C, at an m.o.i. of 0.2 to *E. coli* K12(λ) at a concentration of 5×10^7 bacteria ml⁻¹. After 10 and 20 min, adsorption was stopped by dilution followed by low-speed centrifugation. Samples from the pellet and the supernatant were counted in a scintillation counter. The average results of 10 and 20 min adsorption are expressed as radioactivity in the pellet as a percentage of total recovery. Unspecific adsorption, as measured on *E. coli* B/4, was subtracted for each point. ▲, p.f.u.; ●, radioactivity.

inactivation or autodigestion of trypsin could not explain why the reaction did not proceed, since addition of fresh enzyme after 1 h did not restart it (data not shown).

Inactivation affects adsorbability. Studies of the adsorption of radioactively labelled phages are hindered in two ways: first, only some of the physical particles adsorb to bacteria; second, in our particular case, incubation at 60 °C could lead to non-specific adsorption. Hence, correction has been made for these two factors by considering only the particles able to adsorb before any treatment and by subtracting the background of non-specific adsorption as measured on *E. coli* B/4 (which is resistant to T4). After correction of the raw data, there is good agreement between the kinetics of p.f.u. inactivation and the kinetics of loss of the adsorption properties, at least during the first 30 min of trypsin treatment (Fig. 2).

It has been demonstrated that glucose and glucose-containing oligosaccharides with a (1→3)- α -glucose-glucose linkage hinder the adsorption of phages to bacteria; this is taken as a demonstration of the kinship of this sugar with a bacterial receptor component (hence the adsorption of phage in the presence of lactose and the non-adsorption in the presence of sucrose: Dawes, 1975). The effect of sucrose on trypsin inactivation was therefore studied. Sucrose inhibited trypsin inactivation at the same concentration that it inhibited adsorption to bacteria; lactose did not inhibit (Table 5). This suggests that the target site for inactivation is not far from the specific adsorption site at the tip of the long tail fibres.

Two more instances showed the loss of ability to adsorb to bacteria: quantitative electron microscopy showed that, after inactivation, phages no longer adsorb to bacteria (M. Wurtz, personal communication), and the tip of gp 37, which is known to be the adsorption-specific region, was 'clipped' during inactivation (see below).

These experiments indicate that, during the inactivation reaction, the wild-type phage loses the ability to adsorb to bacteria.

Effect of trypsin on phage proteins. Inactivation was paralleled by partial proteolysis of the tail fibre protein gp 37: SDS-PAGE demonstrated the progressive 'clipping' of this protein

Table 5. *Effect of sucrose on phage inactivation*

Trypsin inactivation was carried out under the standard conditions at various concentrations of sucrose. In a parallel experiment, the action of the same concentrations of sucrose on the adsorption to bacteria was checked: phage suspensions were incubated for 10 min at 37 °C with 10^9 bacteria ml^{-1} , then centrifuged at low speed. The fraction of phage surviving is given for the trypsin treatment experiments and the fraction of phage in the supernatant for the adsorption experiments.

Concn of lactose or sucrose (% w/v) . . .	0	0.75	1.5	2.25	3	6	7.5	15
Sucrose								
wt: trypsin treatment (30 min)	2×10^{-3}	0.02	0.06	0.18	0.3	0.6	0.8	0.9
wt: trypsin treatment (60 min)	10^{-3}	2×10^{-3}	5×10^{-3}	0.02	0.1	0.6	0.5	0.9
<i>ptr</i> : trypsin treatment (30 min)								1.3
<i>ptr</i> : trypsin treatment (60 min)								1.0
wt: adsorption	0.01	0.04	0.01	0.06	0.03	0.09	0.44	0.42
<i>ptr</i> : adsorption	5×10^{-3}	0.04	0.08	0.01	0.2	0.6	0.7	0.6
Lactose								
wt: trypsin treatment (60 min)	10^{-4}	10^{-4}	10^{-4}	10^{-4}	7×10^{-5}	2×10^{-4}	10^{-4}	4×10^{-3}
wt: adsorption	2×10^{-3}							4×10^{-3}

Fig. 3. Trypsin proteolysis of phage particles demonstrated by SDS-PAGE. Sucrose-purified phage particles were treated with trypsin under the standard conditions for 5, 20, 60 and 120 min. The proteins were separated by SDS-PAGE [running gel 10% (w/v) acrylamide] and stained with Coomassie blue. Track D shows the effects of omitting trypsin and adding 5 μg crude DNAase (which contains a protease) ml^{-1} . The gp 37 band of the wild-type disappeared, whereas the corresponding band of the *ptr* mutant did not. (Trailing was due to the absence of DNAase.)

during incubation with trypsin at 60 °C (Fig. 3). The gp 37 band was the only one that was degraded in the wild-type but was not altered in the *ptr* mutant. This clipping gave rise to a fragment about 4–7 kilodaltons smaller than the original gp 37 band, taking the other T4 bands as a calibrating reference. I propose the notation 37*° for this band. The degradation of 37*° was slow. SDS-PAGE of trypsin digests was made possible only by using soya bean trypsin

Fig. 4. Kinetics of gp 37 degradation. Phage particles were labelled by adding [^{35}S]methionine at a final concentration of $5\ \mu\text{Ci ml}^{-1}$ 20 min after infection. The cultures were supplemented with 1% magnesium sulphate 2 min before harvesting, at 90 min. Phage particles were purified on sucrose gradients and treated with trypsin under the standard conditions. Proteins were separated by SDS-PAGE, detected by autoradiography, and quantified by densitometry. The figure is a semi-logarithmic plot of the densitometric measurements of the gp 37 band. Since no gp 37* can be seen at $t = 0$, the value at 120 min has been taken as the standard for this band. The wild-type gp 37 disappeared with first-order kinetics with average $k = 0.045\ \text{min}^{-1}$. The gp 37 of the *ptr* mutant was not digested. Densitometry: ●, gp 37 of wild-type; ○, gp 37* of wild-type; △, gp 37 of *ptr* mutant. The logarithm of the fraction of surviving wild type phages is also shown (□).

inhibitor, since the trypsin proteolysis was not stopped by dilution and since the enzyme remains active in the presence of SDS (Cleveland *et al.*, 1977). The clipping was found with crude lysates as well as with particles purified on sucrose gradients, and it was found with unlabelled preparations (Fig. 3) as well as with radioactive ones (Fig. 4). Clipping could also be seen on long tail fibres purified on sucrose gradients: in this case, the gp 34 band was degraded in the same way in wild-type and in *ptr* particles (data not shown). One can assume that a sensitive site is unmasked in isolated fibres, but masked when these fibres are attached to the base plate by their proximal end, which is made of gp 34.

Degradation of gp 37 was measured by densitometry using a Joyce Loebl spectrophotometer. Semi-logarithmic plots show that the results could fit with first-order inactivation kinetics; the average k for the values in Fig. 4 is $k = 0.045\ \text{min}^{-1}$ (s.d. 0.018). Within the limits of accuracy of the measurements, I have no evidence for a shoulder at the origin of the curve. Taking these two results at face value, there would be no precursor-product relationship between the two phenomena: clipping and p.f.u. inactivation are two distinct reactions. SDS-PAGE of the *ptr* mutant demonstrated that gp 37 was stable during trypsin incubation; however, some protein bands disappeared during the incubation of purified particles of both wild-type and *ptr* strains. As infectivity was preserved in the *ptr* mutant, these bands can be ascribed to dispensable proteins (Fig. 3). Electron microscopy showed the clipping of the tip of the long tail fibres after treatment of the wild type; this clipping was not seen after treatment of a *ptr* mutant (M. Wurtz, personal communication).

Demonstration that the mutation lies in gene 37

Complementation mapping. Attempts to complement a *ptr* mutant with the wild-type failed, because the inactivation curve could not be distinguished from that of the *ptr* mutant alone (Fig. 6). The mixing of the two lysates gave the same result (Fig. 5).

Fig. 5. Complementation of *ptr* phage by wild-type phage. The *ptr* strain was complemented by the wild-type at a constant m.o.i. of 10. The ratios of *ptr* input to total input were 1/2, 1/21, and 1/201. On the other hand, *ptr* and wild-type were mixed in the same proportion: 1/2, 1/21, and 1/201. Trypsin inactivation was carried out under the standard conditions for 20, 40, 60, 100 and 120 min. A semi-logarithmic plot of the surviving fraction as a function of incubation time is presented. The results are plotted after subtraction of the logarithm of the average inactivation of the relevant *ptr* control. The horizontal part of each curve is the average of the last three points. The arrows point to the position of the plateau expected from theoretical considerations. ○, *ptr* control; □, wild-type control, corrected; ●, complemented phages; △, mixture of phages of the two phenotypes.

Fig. 6. Complementation of amber double mutants by wild-type. Double mutants *ptr-am x* (the amber mutation being located in genes 36, 37, 38) were complemented by wild-type T4 on the non-permissive strain *E. coli* B^E. The progeny were examined for trypsin sensitivity as described previously. Each point is the average of three experiments.

Rough localization by crossing. The *ptr* mutant was crossed with amber mutants scattered along the T4 genome. After intermediate culture at low multiplicity, the genotype of 100 single isolated plaques was examined in two different ways: (a) trypsin treatment before plaque isolation and further determination of the *am/am*⁺ genotype by spot tests; (b) isolation of single plaques, followed by resuspension in liquid medium and direct determination for each plaque, by trypsin treatment of each suspension, of the *am/am*⁺ genotype and of the *ptr/wt* genotype. These two experiments were performed on the progeny of 11 crosses (Table 1). The smallest number of recombinants of the type *ptr-am* (6% of the parent *ptr-am*⁺) was obtained from the *ptr* × 37 (*am* B 280) cross. This figure differs significantly both from the expected average result (50%) and from the measured average (61% and 40%, respectively). Thus the *ptr* mutation is located close to gene 37.

A more accurate location of the mutation in relation to gene 37 needed a different approach, which was to complement wild-type phages by double-mutant phages carrying *ptr* and different *am* mutations. The rationale of the experiment is that if the two mutations are located in the same gene, the *ptr* character cannot be expressed, since the whole gene product is not made.

Isolation of double mutants. Mutants 36 (*am* E 1) and 37 (*am* B 280) were by-products of the preceding experiment. In the other crosses, single *am* mutants in the tail fibre region were crossed with *ptr*-23 (*ts* H 86). After intermediate culture at low multiplicity and proteolysis under the standard conditions, the progeny were plated on *E. coli* CR63 (permissive for amber) and incubated at 42 °C. Single plaques were collected and tested for their *am/am*⁺ and *ptr/wt* character by spot tests as described above. The following double mutants were constructed: *ptr* and 37 (*am* N 52); 37 (*am* B 34); 37 (*am* NG 475); 38 (*am* B 262); 38 (*am* A 470); 38 (*am* H 41). I obtained only one *ptr*-37 (*am* NG 475) mutant after screening 150 plaques, and no *ptr*-37 (*am* NG 187) mutants, despite examining 800 single plaques. Neither was such a strain isolated from direct selection after repeated trypsin treatment of the original amber strain.

Complementation of the wild-type by double mutants. Complementation was done in the usual way. The result of the trypsin treatment of the offspring permitted classification of the complemented phages into three groups (Fig. 6):

1. as sensitive as the wild-type: 37 (*am* B 280); 37 (*am* NG 475)
2. as resistant as *ptr*: 38 (*am* B 262); 38 (*am* A 470); 38 (*am* H 41)
3. showing intermediate behaviour: 36 (*am* E 1); 37 (*am* B 34); 37 (*am* N 52).

Taking into account the well-documented polarity of genes 36, 37 and 38 of T4 (Beckendorf, 1973; King & Laemmli, 1971; Stahl *et al.*, 1970; Terzaghi, 1971; Ward & Dickson, 1971) the above data are only compatible with two explanations: that the *ptr* mutation maps in gene 37 or that the *ptr* mutation maps in a new gene located between genes 37 and 38.

ptr does not map in a new gene between genes 37 and 38. To eliminate the possibility that an as yet unidentified gene between 37 and 38 might carry the *ptr* mutation and be unexpressed owing to the polarity 37, 38, I isolated revertants of the *am* in gene 37 which were *ts*. NG 475 gave the same response as *am* or as the *ts* revertant (Fig. 7). This eliminates the possibility that the expression of a new gene may be hindered by an amber mutation in gene 37 because of the polarity of the operon.

Tentative fine localization inside gene 37. To obtain better information on the location of *ptr*, I made three-factor crosses of *ptr*-37 (*am* x) × 38 (*am* B 262) (*am* B 262 is the closest mutation to the amino-terminal end of the known 38 *am*). After intermediate culture at low multiplicity, I examined the trypsin resistance of isolated plaques of *am*⁺ recombinants. The results (Table 6) provide evidence for the location of the *ptr* mutation near the carboxy-terminal end of gene 37 and probably close to the marker NG 475.

DISCUSSION

The properties and assembly of the long tail fibres of T4 have been extensively studied (for reviews, see Wood & Bishop, 1973; Wood *et al.*, 1972). The remote part of the distal arm of the long tail fibres, where the mutation(s) studied in this paper map(s), has the property of recognizing bacterial receptors (Simon & Anderson, 1967; Prehm *et al.*, 1976). It has many

Fig. 7. Complementation of thermosensitive double mutants by wild type. Double mutants *ptr* and *ts* 37 were isolated as revertants of the amber mutant as described; under non-permissive conditions (41 °C), they were complemented by wild-type T4 in an experiment similar to that described in Fig. 6. *ts* revertants are named by the amber they come from. The thermosensitive revertant from 37 *am* NG 475 is as susceptible to trypsin as the wild-type. Each point is the average of three experiments.

Table 6. *Three-factor cross*

Several double mutants carrying the *ptr* mutation and different *am* mutations in gene 37 were crossed with 38 (*am* B 262). After intermediate culture at low multiplicity, the trypsin resistance of isolated plaques of *am*⁺ recombinants was examined.

<i>am</i> mutant . . .	36 (<i>am</i> E 1)	37 (<i>am</i> B 34)	37 (<i>am</i> N 52)	37 (<i>am</i> B 280)	37 (<i>am</i> NG 475)
<i>am</i> ⁺ - <i>ptr</i> /wt total	85/200	56/100	55/200	19/99	9/100

peculiarities and this quarter of gp 37 (which includes the carboxy-terminal end) does not have the morphological, biological and biophysical properties of the rest of the molecule (Beckendorf, 1973; Beckendorf *et al.*, 1973). Recent DNA sequencing of gene 37 does not invalidate Beckendorf's discoveries and the hypotheses derived from them (Oliver and Crowther, 1981).

The inactivation reaction. The reversibility of temperature action (Table 3) associated with the abrupt thermal transition (Table 4) strongly suggests the involvement of reversible protein denaturation. Conversely, protease inhibition during T4 infection has been described (Simon *et al.*, 1978). Beckendorf (1973) has proposed a γ -helical structure for the last quarter of the distal arm. However, this author pointed out that the structure lacked the instability predicted for this conformation. Demonstration of thermal instability would confirm Beckendorf's hypothesis.

Clipping gives rise to only one band on gels (Figs 3 and 4). This means that the two polypeptide chains constituting the distal arm (Ward & Dickson, 1971) must be cut at the same site, since the two gp 37 are oriented linearly and parallel in the distal arm (Beckendorf, 1973), and Oliver & Crowther's (1981) data do not provide two close sites which could fit our SDS-PAGE measurements. This would also confirm Beckendorf's model. Both the mechanism of denaturation and correct spontaneous renaturation deserve further consideration.

Another surprising observation is the difference between the slope of the p.f.u. inactivation curve and that of the SDS-PAGE clipping curve (Fig. 4). Since the *ptr* mutation, located in gene 37, prevents p.f.u. inactivation, I am led to suspect that trypsin provokes an inactivation that escapes detection by SDS-PAGE. Only Arg 1024 of Oliver & Crowther's (1981) sequence can fit the data: the reaction would liberate a dipeptide, Leu-Ala-COOH, an hypothesis worth testing. The other site of denaturation, with a probability ten times lower, could be at Lys 945 or

Arg 954. These two different probabilities of denaturation are good arguments in favour of at least two distinct mutations, as will be discussed below.

Another unanswered question is the reason why the curves presented here demonstrate first-order kinetics in spite of the fact that phage T4 has six long fibres, three of which are necessary for phage adsorption (Wood & Henninger, 1969; Crawford & Goldberg, 1980): one would expect at least a three-hit phenomenon. Several hypotheses could explain the observation, among which I should mention: (1) the formation of a new, sensitive, cooperative structure at 60 °C; (2) that the clipping of only one fibre could perturb the reversible step of adsorption (Crawford & Goldberg, 1977); (3) that three fibres only are normally involved in phage T4 adsorption. Further studies are necessary to distinguish between these hypotheses.

Genetical results. Genetics is not easy because of the complexity of the region of the genome coding for tail fibre proteins, the polarity of the 36, 37, 38 region and, in the case of my mutant, the phenotypic dominance. The differences in behaviour of gene 37 double mutants deserve some comment. The intermediate sensitivity of the offspring of coinfection by wt and *ptr*-36 (*am* E 1) is clearly explained by polarity. On the other hand, the same phenomenon is less easy to understand for *am* B 34 and *am* N 52, since the mutants are not leaky. I compared the trypsin sensitivity of phage particles at 30 min and 60 min after coinfection (data not shown): the differences were too small to explain the differences in trypsin sensitivity. Polarity does not explain the results, since the closer to the carboxy-terminal the mutants are, the more sensitive the offspring. An explanation can be found either in abnormalities of recombination (which have been described in this region: Fisher & Bernstein, 1970) or in intragenic complementation, of which gp 37 provides one of the best instances (Bernstein *et al.*, 1965).

Several facts suggest that my *ptr* mutant might carry more than one mutation. First, the way it has been selected implies that it is not a single-step mutant. The different slopes for p.f.u. inactivation and gp 37 clipping suggest two different, distant, regions of denaturation. Secondly, the behaviour of some mutants in genetical experiments argues in favour of a double mutation: particularly the observation that the double mutant *ptr*-*am* B 280 gave the sensitive response, even though the *ts* derived from it did not.

In conclusion, the *ptr* mutation(s) map(s) in gene 37, but accurate numbering and location of the mutation(s) await DNA sequencing of this part of gene 37 of the *ptr* mutant.

The experimental observations contribute to the outlining of two biological entities, each being part of the definition of the other: a specific inactivation reaction and the corresponding resistant mutant.

The practical interest of the *ptr* mutation is obvious: it suggests a new way of purifying phage T4 free of dispensable proteins; it produces a new frame of reference for the definition of the electrophoretic pattern of T4 proteins, and indicates which are minor and which dispensable proteins; it provides a means of detection of any protease active at 60 °C. From the theoretical viewpoint, I feel the most surprising fact is that modification of a single protein causes phage inactivation under the conditions defined in this paper.

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