INFECTION-TRIGGERED RELEASE OF TEMPOCHOLINE FROM BACTERIOPHAGE T4 STUDIED BY ELECTRON SPIN RESONANCE

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1. Introduction

The complex infection process of bacteriophage T4 involves a step of DNA ejaculation into the host bacterium, *Escherichia coli* [1]. DNA 50 μm in length packaged inside the head shell (1100 Å length, 850 Å diam.) passes through the cylindrical tail tube. Accompanying the DNA, internal proteins are also released into the bacterium [2]. We have found that a spin label tempocholine (*N,N*-dimethyl-*N*-(2′,2′,6′6′-tetramethyl-4′-piperidyl)-2-hydroxyethylammonium chloride) can be incorporated in the heads of phage T4 and is rapidly released when phage are mixed with bacteria or bacterial envelopes. This infection-triggered release of tempocholine was monitored by the electron spin resonance (ESR) method and its kinetics seem to parallel those of DNA ejaculation.

2. Materials and methods

2.1. *Bacteria and phage*

Escherichia coli B^E were grown at 37°C in M9s medium, containing per liter: 7 g Na_2HPO_4; 3 g KH_2PO_4; 0.5 g NaCl; 1 g NH_4Cl; 1 mM $MgSO_4$; 0.1 mM $CaCl_2$; 4 g glucose; 10 g casamino acids (Difco). Bacteria grown to 5×10^8/ml were collected by centrifugation and resuspended in M9 buffer (M9s without glucose and casamino acids). Bacteria were disrupted by sonication and the pellets washed by M9 buffer were used as the envelope fraction.

Phage strains used were T4D and T4B wild-type, T4D*os*, T4D*12*(amN69) and T4D*soc*(sd50) [3] mutants.

For adsorption experiments, freshly prepared bacteria (10^9–2×10^{10}/ml) or envelopes of equivalent concentrations in M9 buffer were mixed with purified phage at ~ 50–200 multiplicity.

2.2. *ESR measurements*

Procedures of ESR measurements are similar to those in [4]. ESR spectra were measured with a commercial X-band spectrometer (JEOL Model ME 2X). In order to follow rapid kinetics of the tempocholine release, the samples were mixed with a stopped-flow apparatus (Union Giken MX-7) and lead to the cavity. Tempocholine chloride was synthesized as in [5]; m.p. 221–224°C.

Incorporation of tempocholine into phage was determined as follows: T4D wild-type phage (final conc. 4 mg/ml as protein determined by Biuret method [6]) were suspended in 140 mM tempocholine solution in 10 mM Tris–HCl, at pH 7.6 and incubated at 37°C for various times. The suspension was centrifuged at 13 000 rev./min at 2°C and the pelleted phage were washed 3 times with M9 buffer. The amount of incorporated tempocholine was determined from the peak height of the ESR spectrum after heat-treatment of the phage suspension at 70°C for 10 min.

The release of tempocholine from phage was measured in situ by an increase in the peak height of ESR spectrum of phage loaded densely with tempocholine. The spectrum was broadened by the spin–spin exchange interaction and, when tempocholine was released, the spectral lines became narrower and the peak height increased because of disappearance of the exchange interaction due to dilution (fig.2B).

3. Results and discussion

As shown in fig.1, the penetration rate of tempocholine was slow and the incorporated tempocholine was almost saturated after 18 h at 37°C. The ESR spectrum of the labeled phage showed a considerable exchange broadening (fig.2A, solid line) and the apparent concentration of tempocholine in the phage was estimated to be about 40 mM. The mobility of tempocholine is somewhat restricted (rotational correlation time τ_c 5×10^{10} s), perhaps due to interaction with the phage components; τ_c of free tempocholine is $\sim 3 \times 10^{11}$ s. Penetration kinetics into head mutants, T4*soc* and T4*os*, were also studied. The rates of incorporation were indistinguishable from that of T4 wild-type, suggesting that permeability of tempocholine through the head membrane is not significantly altered in these mutants.

T4 ghost phage (heads emptied by repeated freeze–thawing) were incubated with tempocholine under the condition described above, and a much lower concentration of tempocholine ($\sim$ 1.5 mM) was found to remain after washing 3 times. This is probably due to leakage of tempocholine from the emptied heads. ESR spectra of the remaining tempocholine showed little exchange broadening effect and its $\tau_c \sim 1 \times 10^{11}$ s, indicating that tempocholine inside the particles moves relatively freely (fig.2A, broken line). The bulk

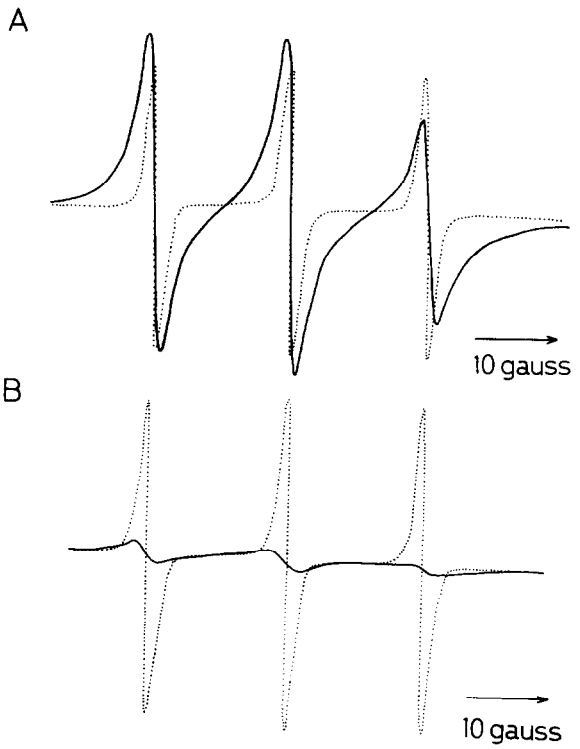

Fig.2. A. ESR spectral profiles of tempocholine-loaded T4 phage (solid line) and T4 ghost (broken line). The scale of peak heights is arbitrary. B. ESR spectra of T4 phage suspensions before (solid line) and after (broken line) mixing with *E. coli*. A sharp increase of peak heights was observed after mixing with bacteria.

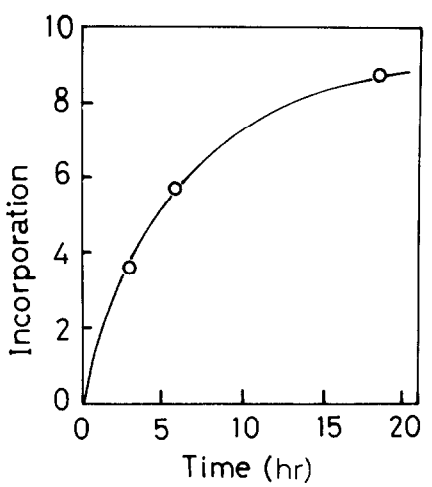

Fig.1. Penetration of tempocholine into T4 phage. The scale for tempocholine incorporation is only relative.

of the tempocholine incorporated into T4 phage appears to be present in the phage head. Approx. 50% of the physical volume of the head is occupied by the packaged DNA. If the packaged DNA winds up in the whole volume of the head, the space occupied by tempocholine should consist of folded regions rather than a single domain (e.g., a center hole). Such folded regions may affect the motion of tempocholine.

Spontaneous release of tempocholine from phage is slow at 37°C (fig.3, broken line). At 2°C it is much slower. On the other hand, tempocholine leaks rapidly at 70°C for 10 min. The difference in the peak heights of ESR spectra between the heat-treated and untreated phage suspensions is about 9 fold. In the following experiments, extents of tempocholine release are expressed as percentage (100% equivalent to peak height of heat-treated phage).

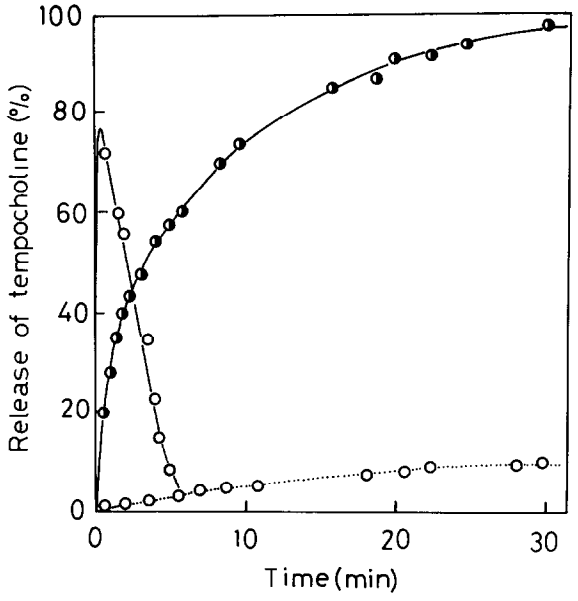

Fig.3. Release of tempocholine from T4 phage at 37°C when they were incubated alone (- - - o - - -) or with bacteria (——o——) or bacterial envelopes (——●——).

Figure 2B shows ESR spectra of T4 phage before and after mixing with bacterial envelope. The striking change in the spectral profile indicate the release of tempocholine. Figure 3 shows the time course of tempocholine release when T4 phage (2×10^{12}/ml) were incubated at 37°C with freshly-prepared bacteria (2×10^{10}/ml), bacterial envelopes and without bacteria. On mixing with bacteria, the amount of released tempocholine rapidly reached a maximum within 1 min and then it appeared to fall off. However, if the bacterial concentration was reduced 10 fold, the rate of tempocholine release was reduced and the decrease after the maximum was negligible (data not shown). The fall-off is due to reduction of tempocholine by living bacteria. On the other hand, tempocholine was also released but with much reduced rate compared with whole bacteria when they were mixed with the envelope fractions. The release reached a maximum at around 30 min. No reduction of the tempocholine radical group was observed.

Phage T4B which requires tryptophan for adsorption released tempocholine rapidly only under the presence of tryptophan, when mixed with bacteria (data not shown). Therefore, the tempocholine release is triggered by adsorption of phage to the bacterial surface.

Defective phage lacking the baseplate protein P12 are unable to inject DNA into bacterium; they can adsorb and contract the tail sheath but then fall off from the bacterial [7]. Figure 4 shows the time course of tempocholine release by defective 12^- phage when mixed with envelopes, in comparison with T4 wild-type. The amount of the released tempocholine from 12^- phage was less than half the wild-type, indicating that the bulk of the incorporated tempocholine remained in 12^- phage. Thus, unless the DNA ejaculation occurred, the phage with contracted tails still contained tempocholine. Although only full heads with contracted tails were reported to be present after mixing 12^- phage with bacteria [7], significant number of empty heads with contracted tails were observed in our preparation. This may be due to the effect of tempocholine and probably represents the minor release of tempocholine from 12^- phage.

Experiments were carried out to analyze the rapid kinetics of tempocholine release by the stopped-flow

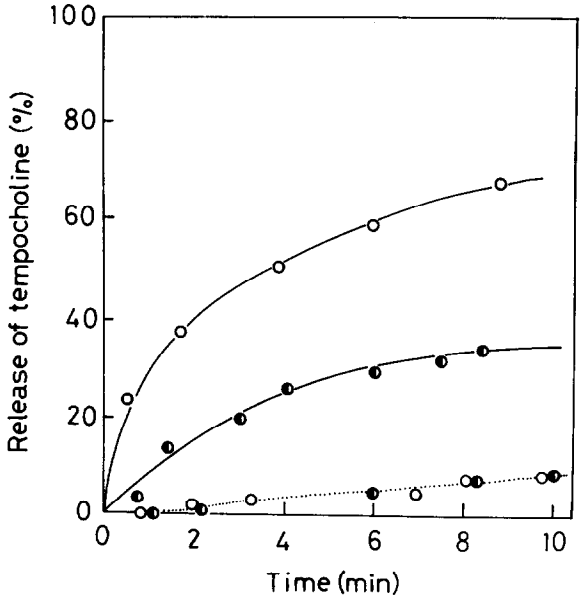

Fig.4. Release of tempocholine from 12^- phage (——●——) and wild-type phage (——o——) mixed with envelopes. Broken lines represents the tempocholine release without envelopes.

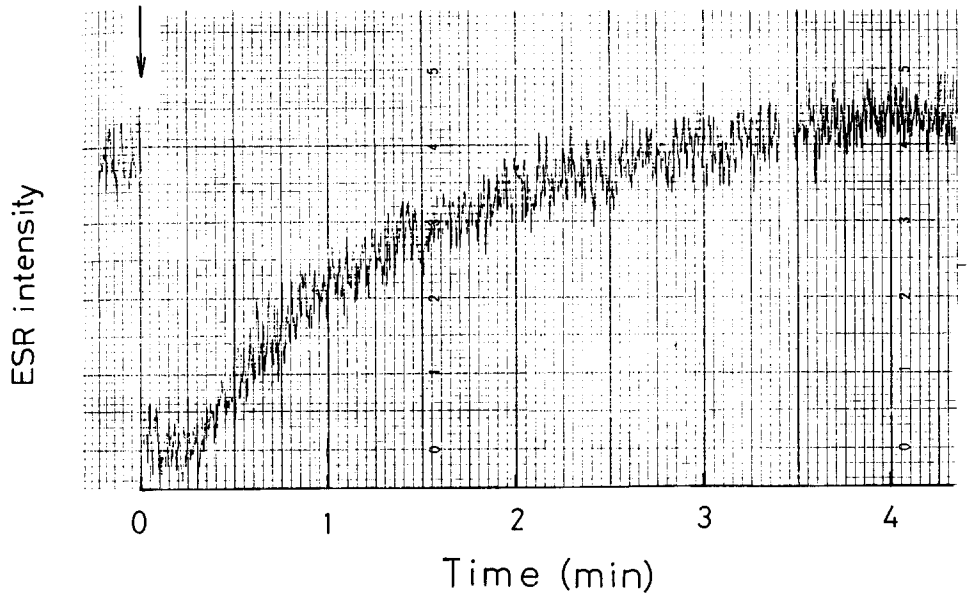

Fig.5. Time course of the tempocholine release from T4 phage mixed with envelopes by the stopped-flow method.

method. Figure 5 shows the time course of tempocholine release at 22°C by T4 phage mixed with envelopes. A lag of about 20 s is clearly observed before the initiation of tempocholine release. The lag may represent a step of reversible adsorption in which tail fibers are not attached to the bacterial surface. We conclude that the kinetics of tempocholine release are closely related to those of DNA ejaculation.

In short, our results show that tempocholine is a useful probe inside the head and that the ESR method combined with the stopped-flow technique should be a powerful tool for a detailed analysis of the mechanism of the phage infection process.

Acknowledgement

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