Purification and Characterization of the Protein Product of Gene 11 of Bacteriophage T4D*

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SUMMARY

The protein product of gene 11 of bacteriophage T4D was purified to apparent homogeneity from a bacteriophage culture in which this particular structural protein had not yet become incorporated into the mature bacteriophage particle. Monomer and apparent trimer molecular weights were determined to be approximately 24,000 and 70,000, respectively, and an amino acid analysis was performed. The kinetics and stoichiometry of interaction between the product of gene 11 and 11− defective bacteriophage particles were examined.

In the study of the molecular details of the assembly of biological structures, a potentially useful approach is to isolate the relevant macromolecular components after they have been synthesized but prior to incorporation. With such material in hand, one is then in a position to study, by means of the many physical and chemical techniques currently available, the precise mode of interaction which leads to the biologically meaningful macromolecular complex.

The coliphage T4 system is particularly well suited for this sort of an endeavor because it is of an appropriate degree of structural complexity, its genetics have been very extensively studied, and the structural function of many genetically identified gene products have been described (1) as have the pathways by which gene products sequentially interact with one another to give the final virus structure (2). From the point of view of purification of structural proteins, again the T4 system is well suited because judicious use of amber mutations permits the production of selectable gene products prior to incorporation. With such material in hand, one is then in a position to study, by means of the many physical and chemical techniques currently available, the precise mode of interaction which leads to the biologically meaningful macromolecular complex.

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E. coli B was grown to $4 \times 10^8$ bacteria per ml in M9a at 37°C and infected with a multiplicity of 5 bacteriophage per bacterium (moi = 5) and superinfected 5 min later (moi = 5) to assure lysis inhibition. Starting at about 60 min, the infected bacteria were harvested without chilling, by continuous flow centrifugation. The yield was approximately 2 liters of a very thick slurry of infected bacteria. Phage heads were recovered from this preparation by freeze-thaw lysis followed by a series of differential centrifugations culminating in centrifugation in a Spinco type 30 rotor for 2 hours at 24,000 rpm. The P11 starting material was the supernatant from this final centrifugation.

At the end of P11—The titrations of this protein whose function appears to be purely structural, depends on its ability to activate, in vitro, the phage particles in a gene 11 defective lysate and convert them into apparently normal, active phage particles. To generate the requisite gene 11 defective lysate, a culture of E. coli B was grown in M9a at 37°C with aeration to a titer of about $10^8$ the initial volume of fresh M9a medium containing a minute amount of DNase, egg white lysozyme (1 mg per ml), and a few drops of chloroform. Aliquots (1 ml) of the resulting slurry were distributed to a series of tubes and stored frozen. When needed, a tube was thawed and used as described below.

To assay for P11, aliquots (usually 0.05 ml) of the defective lysate were distributed to a series of tubes, and to these were added equal aliquots of, for example, fractions from a column. The tubes were sealed with Parafilm and then incubated overnight (unless stated otherwise) at 37°C. The incubation mixtures were then diluted with 10-ml dilution buffer and active bacteriophage titrated on E. coli C663. Titers refer (unless otherwise stated) to the concentration of bacteriophage in this initial dilution tube. The sensitivity and proportionality of this assay will be discussed in subsequent sections of this report.

Chromatography—Whatman DE52 cellulose, Pharmacia Sephadex G-200, Bio-Gel P-150, and Bio-Gel P-300 were used according to the instructions supplied by the manufacturer. Calibration of the Sephadex and Bio-Gel columns was carried out with blue dextran (excluded volume) and bromphenol blue (included volume) and the molecular weight standards of egg white lysozyme, myoglobin, trypsin, and bovine serum albumin. In the elution profiles presented, optical densities were measured in the usual way with 1-cm cuvettes in a Zeiss spectrophotometer; the P11 activities are the raw complementation data corrected only for the appropriate dilution factors. Buffer concentrations were determined with a homemade conductivity meter.

Buffers—For the cellulose chromatography, the buffers routinely used were 0.01 M, 0.1 M, and 0.25 M potassium phosphate at pH 7.5 with 0.05% mercaptoethanol and 10^{-3} M MgSO_4 added. The buffer used in the dilution buffer just described was also used for the Sephadex chromatography: a 0.1 M potassium phosphate buffer at pH 6.0 with $10^{-4}$ M MgSO_4 added was used in the Bio-Gel P-150 chromatography and 6 M urea in 0.1 M potassium phosphate buffer at pH 6.3 with $10^{-3}$ M MgSO_4 added was used in the Bio-Gel P-300 chromatography.

Sodium Dodecyl Sulfate Gel Electrophoresis—SDS\(^8\) gel analyses were done on 5% polyacrylamide gels in 0.1% SDS and 0.1 M sodium phosphate, pH 7.2, as a test of homogeneity and for a crude molecular weight determination. All samples and standards were pretreated for 10 min in pH 7.2, 0.01 M sodium phosphate, 0.1% SDS, and 0.14 M mercaptoethanol at 60°C just prior to application to the top of the gel (neither stacking nor sample gels were used); the runs were carried out at room temperature with 40 V and approximately 8 mA per tube. Staining was done overnight with 0.25% Coomassie brilliant blue in methanol-acetic acid-water (5:1:5); electrophoretic destaining was carried out in 7.5% acetic acid.

Ultracentrifugation—The Spinco model E analytical ultracentrifuge, equipped with schlieren optics, was used for a sedimentation analysis according to the instructions provided with the machine.

Amino Acid Analysis—Samples for analysis were dried in vacuo over P_2O_5, resuspended in 0.5 ml of 8.7 N HCl (distilled), sealed under reduced pressure, and hydrolyzed at 106°C for the indicated periods of time. After hydrolysis, the HCl was rapidly removed under reduced pressure at 80°C. Analysis was carried out on a Beckman-Spinco amino acid analyzer, model 120C, equipped with an expanded range recorder and long pathlength cuvettes, according to instructions provided with the instrument. The tryptophan content was estimated spectrophotometrically according to the technique of Beaven and Holiday (12) and the half-cystine content was examined by amino acid analysis of a sample of P11 that had been perform oxidized at $-10^5$ according to the method of Hirs (13).

RESULTS

Assay

When a dilution series of P11, whether in the form of a crude defective lysate or in a relatively pure state, is assayed, an approximately second order relationship is found between concentration and titratable activated bacteriophage. For any given 11- defective lysate, there is a clearly defined P11 concentration at which point the system is saturated, and greater P11 concentrations do not lead to higher titers of activated bacteriophage particles (Fig. 1). However, in order to keep the presentation of results as simple as possible, the "activity" portion of elution profiles shall be reported in terms of activated bacteriophage titers.

As there are very substantial differences between different preparations in respect to the efficiency with which they are activated (particularly between fresh and frozen defective lysates), direct quantitative comparisons between different elution profiles may not be made.

Fig. 1. Standard dilution curves of P11 of different origins. Three 2-fold dilution series of P11 were assayed with gene 11 defective lysates as described under "Materials and Methods": (---) represents the results obtained from a purified sample of P11, whose $A_{280}$ = 0.05 in the most concentrated aliquot; (---) represents the results obtained from a crude, concentrated defective lysate of 7(amN128); and (X--X) represents the results obtained from a crude, concentrated defective lysate 10(amB255). The later two defective lysates were prepared identically, in parallel, and accordingly represent the P11 yield of roughly equivalent titers of Escherichia coli B infected by, respectively, gene 7 and 10 amber mutants.

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1 The abbreviation used is: SDS, sodium dodecyl sulfate.
To keep an approximate account of activity recovery, a sample of the starting material in which P11 appeared to be stable was assayed along with each set of P11 assays; in accord with the apparent second order nature of the activation, the square root of the raw data was taken as a measure of the relative P11 activities of the samples assayed; the recovery was then calculated relative to the starting material.

**PI1 Purification**

An ammonium sulfate fractionation of the PI1 starting material, described under "Materials and Methods," was followed by a series of chromatographic separations, first on DEAE-cellulose and then Sephadex G-200, resulting in a material which appeared homogeneous by the criteria of sedimentation velocity and SDS gel electrophoresis. All steps in the purification were carried out at 4°C.

For each chromatographic step, an elution profile is presented; the activity recovery from the various stages of purification is summarized in Table I.

**Table I Summary of PI1 purification**

Here are summarized quantitatively the material recovered from selected steps in the purification of PI1 of bacteriophage T4. For the purposes of this presentation, "Activities" are given as relative to the starting material, which appeared to be stable and the volume of the material at that step. Recovery is based on the product of the relative activity and the volume of the material at that step. Purification is the quotient of the specific activity at that step and the specific activity of the starting material.

| Material                                | Volume | Amax | Relative activity | Specific activity | Recovery | Purification |
|-----------------------------------------|--------|------|-------------------|-------------------|----------|-------------|
| High speed supernatant                  | ml     |      |                   |                   | %        |             |
| 20-40% ammonium sulfate fraction        | 2600   | 265  | 1                 | 0.0038            | 100      | 0           |
| First DEAE-cellulose column (3.9 x 20 cm) | 560    | 39.5 | 3.7               | 0.094             | 77       | 25          |
| Second DEAE-cellulose column (3.9 x 20 cm) | 280    | 0.82 | 11.5              | 14                | 124      | 3700        |
| Third DEAE-cellulose column (1.3 x 20 cm) | 95     | 0.48 | 6.1               | 12.7              | 21       | 3300        |
| Sephadex G-200 column                   | 75     | 0.34 | 7.4               | 22                | 22       | 5800        |

**Fig. 2. First chromatography of PI1 of bacteriophage T4 on DEAE-cellulose.** The column was prepared, the sample was applied, and gradient elution was carried out as described in the text. Approximately 25-ml fractions were collected. PI1 activities are presented as the raw data of the complementation assays, corrected only by the appropriate dilution factors. ———, the pooled portion used in subsequent steps.
with an $A_{280}$ of 5.2. This final concentrated material was used for subsequent experiments.

**Summary**—Table I summarizes the purification of P11 in terms of relative activities recovered at key steps in the purification scheme. There are several points which deserve mention. (a) A very high recovery is recorded from the first DEAE-column, which may reflect either serious quantitative inadequacies of the P11 assay or purification away from inhibitory materials. This problem has not been further investigated. (b) Judging from the specific activity figures, Steps 3, 4, and 5 add little or nothing to the purity of P11, although succeeding elution patterns improve in quality. Accordingly, one of these steps would be abandoned in future preparations of this protein.

**Characterization of P11**

**Analytical Ultracentrifugation**—An aliquot of the purified preparation at a concentration of approximately 7.3 mg per ml was examined in a sedimentation velocity run in order to determine the homogeneity of the sample and to measure the sedimentation constant. The $s_{20,w}$ value was calculated to be 3.8 S. Runs done with material at $\frac{1}{3}$ and $\frac{1}{4}$ the concentration yielded the same value. Fig. 5 shows the frame taken at 80 min.

**Absorption Spectrum**—P11 yields a characteristic protein spectrum in the region of 250 to 300 nm at pH 7.5, with a $A_{280}:A_{260}$ ratio of 1.9. Amino acid analysis of samples of known absorbance indicated that 1 $A_{280}$ unit corresponds to a concentration of approximately 1.3 mg per ml.

**Gel Electrophoresis**—In order to have a further independent measure of the purity of the final material as well as an indication of its molecular weight, the final material was subjected to SDS gel electrophoresis, along with the molecular weight standards of egg white lysozyme, hemoglobin, trypsin, pepsin, and bovine serum albumin (14). All six were run separately to determine their mobilities under these conditions of electrophoresis and they were all run together on a single gel in order to measure more precisely the mobility of the P11 relative to the others. P11 was found to migrate as a single homogeneous band (Fig. 6A) when run alone, and comigrated with trypsin (Fig. 6B) when run along with the collection of molecular weight standards. The latter observation indicates a monomer molecular weight value of about 24,000.

**Gel Filtration**—To obtain a molecular weight estimate of P11 in its native form (corresponding to the 3.8 S value observed in the sedimentation velocity analysis), a calibrated gel filtration was done on a column (2 x 40 cm) of Sephadex G-200 in 0.01 M potassium phosphate buffer (pH 7.5). Bovine serum albumin and trypsin, in addition to bromphenol blue and blue dextran, were used for calibration of the column. The elution positions of the standards were plotted against the log molecular weight (Fig. 6C); the peak of P11 was found to elute several milliliters in advance of bovine serum albumin, which indicates a native molecular weight in the neighborhood of 70,000 (15).
As P11 activity was not recoverable from the low molecular weight band seen in SDS electrophoresis, the question could be raised as to whether that band indeed represented P11. To investigate this point, a fresh small scale (8 liters) preparation of Bio-Gel P-300 column was made and carried through the ammonium sulfate stage of purification. This material was run directly on a calibrated Bio-Gel P-100 column (2 x 40 cm) equilibrated with 0.1 M potassium phosphate buffer at pH 6.0. A peak of P11 activity appeared which corresponded to the bulk of 280 nm absorbing material eluting in the excluded volume (Fig. 7A). The active fractions were pooled, concentrated, and run on a calibrated Bio-Gel P-300 column equilibrated with 6 M urea in 0.1 M potassium phosphate buffer at pH 6.3. Individual fractions were concentrated, dialyzed to free them of urea, and assayed. The main peak of P11 activity had shifted to a position corresponding to a molecular weight of about 27,000, which was quite distinct from hemoglobin, trypsin, pepepin, and bovine serum albumin, were done individually and in combination. Log molecular weights of the standards are plotted against their relative mobilities. C, a column (2 x 40 cm) of Sephadex G-200 was equilibrated with 0.01 M phosphate buffer (pH 7.5). The elution positions of the molecular weight standards trypsin and bovine serum albumin were separately determined and plotted against log molecular weight. The elution positions of P11, blue dextran and bromphenol blue were also determined and are indicated on the standard curve.

**Fig. 6.** SDS gel electrophoresis and molecular weight determination of P11. A, gel electrophoresis of P11 was done in 5% polyacrylamide gels in 0.1 M sodium phosphate (pH 7.2) containing 0.1% SDS. Electrophoresis was performed at room temperature using approximately 40 volts and 5 ma per tube. Run time was approximately 4 hours. A densitometric tracing of the Coomassie blue stained gel is shown. (The broad low “peak” is an artifact of destaining encountered with all of the gels in this series of runs.) B, SDS gel electrophoresis (as described in A) of P11 and the molecular weight standards of egg white lysozyme, myoglobin, and bovine serum albumin were separately determined and plotted against log molecular weight. The elution positions of the dye markers are also shown. The pooled active samples from the column run described above (A) were concentrated and chromatographed. Fractions were concentrated and dialyzed against the standard 0.1 M potassium phosphate buffer (pH 7.5) and assayed in the usual manner. The major peak of activity eluted in the position indicated.

**Fig. 7.** Molecular weight determination of multimer and monomer P11 activity. A, a column (2 x 40 cm) of Bio-Gel P-150 was equilibrated with 0.1 M potassium phosphate buffer at pH 6.0 and 10°. The elution positions of the molecular weight standards myoglobin and bovine serum albumin were separately determined and plotted against log molecular weight. The elution positions of blue dextran (excluded volume) and bromphenol blue (included volume) are also shown. A crude preparation (20 to 25% ammonium sulfate cut of a 10° defective lysate) was chromatographed on this column and the fractions assayed for P11 activity. B, a column (2 x 40 cm) of Bio-Gel P-300 was equilibrated with 6 M urea in 0.1 M potassium phosphate buffer (pH 6.3) at 10°. The elution positions of the molecular weight standards of egg white lysozyme, myoglobin, and bovine serum albumin were separately determined and plotted against log molecular weight. The elution positions of the dye markers are also shown. The pooled active samples from the column run described above (A) were concentrated and chromatographed. Fractions were concentrated and dialyzed against the standard 0.1 M potassium phosphate buffer (pH 7.5) and assayed in the usual manner. The major peak of activity eluted in the position indicated.
TABLE II
Amino acid composition of P11

The values are recorded as molar ratios based on glycine = 19.2. The average values are the simple mean of the results obtained from three hydrolyses, except where indicated to the contrary.

| Amino acid | Hydrolysis for | Average |
|------------|----------------|---------|
|            | 48 hrs | 72 hrs | 96 hrs |       |
| Lysine     |     10.0 | 9.3   | 10.8   | 10.0  |
| Histidine  |     5.6   | 6.4   | 6.0    | 5.7   |
| Arginine   |     7.8   | 6.4   | 7.7    | 7.3   |
| Aspartic acid | 22.7 | 24.1  | 23.7   | 23.5  |
| Threonine  |     20.9  | 22.9  | 20.9   | 21.6  |
| Serine     |     18.4  | 17.0  | 13.8   | 23.0* |
| Glutamic acid | 20.6 | 22.0  | 19.1   | 20.6  |
| Proline    |     12.0  | 11.1  | 11.3   | 11.5  |
| Glycine    |     19.2  | 19.2  | 19.2   | 19.2  |
| Alanine    |     16.5  | 15.9  | 14.7   | 15.7  |
| Half-cystine |      0   | 0     | 0      | 0*    |
| Valine     |     17.0  | 17.8  | 17.9   | 17.9  |
| Methionine |      0    | 0     | 0      | 0*    |
| Isoleucine |     17.0  | 15.4  | 16.9   | 16.4  |
| Leucine    |     15.2  | 13.1  | 15.0   | 14.4  |
| Tyrosine   |      7.2  | 6.1   | 5.8    | 6.4   |
| Phenylalanine | 12.5 | 10.4  | 11.3   | 11.4  |
| Tryptophan |      1.9  |      |        | 1.9*  |
| Total      |       |       |        | 226.5 |

* The value for serine was obtained by back extrapolation to zero hydrolysis time.

† The values for half-cystine and methionine were confirmed by analysis of a performic oxidized sample of P11.

‡ Tryptophan was estimated spectrophotometrically with a sample of unhydrolyzed P11 in 0.5 N KOH.

The one striking feature of the amino acid composition of this protein is the apparent complete absence of the sulfur-containing amino acids, cysteine and methionine. This observation was confirmed in an analysis of a performic-oxidized sample of this protein.

Kinetics and Stoichiometry of Interaction of P11 and 11-Defective Particles—A 2-fold dilution series of P11 was made, covering a concentration range of greater than 10⁸, and the kinetics of activation was measured with each aliquot in the series. In Fig. 8A are presented three characteristic curves observed with, respectively, concentrated, moderately dilute, and very dilute P11. In Fig. 8B are plotted (as a function of relative P11 concentration) the maximum rates of activation and the end points, respectively. In the lower portion (more dilute P11) of both curves, a 1.5 order concentration dependence is observed. The end point (72-hour activation) bacteriophage titer level off at about 1.4 X 10¹⁰ bacteriophages per ml, at a P11 concentration of about 5 X 10⁻⁴ mg per ml. Particle titration of the defective lysate by electron microscopy yielded a comparable figure (9 X 10⁹).

**DISCUSSION**

The purification of P11 of bacteriophage T4 has been described, as has a few of its physical and biological properties. That the material isolated is physically homogeneous is supported by the production of a single peak on DEAE-cellulose chromatography and Sephadex G-200 chromatography, a single moving boundary in a sedimentation velocity analysis and a single band in SDS gel electrophoresis. The identification of this material as P11 is shown by the Nuclease peak of P11 activity and the peak of optical density in both types of chromatography, and a molecu-
lar weight of about 70,000 has been tentatively assigned to this species on the basis of its elution position in calibrated Sephadex G-200 runs. The sedimentation value of 3.8 S is not inconsistent with that assignment. That the molecular weight deduced from the calibrated SDS gel electrophoresis is 24,000 suggests that the material originally isolated was multimeric, perhaps a trimer. Although we have not yet been able to demonstrate definitively a congruence of Pll activity with purified material behaving physically as 24,000 daltons, two lines of evidence support this contention. Using a crude preparation of P11, we were able to show that, when run on a Bio-Gel P-300 column in 6 M urea, there is a peak of P11 activity corresponding to a molecular weight of about 27,000. Furthermore, experiments of others (16) have demonstrated that when the SDS gel patterns of a 14C-labeled nondefective bacteriophage lysate are compared with those derived from an 11- defective lysate, the latter differs from the former only in the lack of a single band at a position corresponding to a molecular weight of approximately 26,000. Thus we are confident that the single low molecular weight band observed with the purified material in SDS gel electrophoresis is indeed P11.

One chemical feature of this protein worthy of note is the failure to detect the sulfur-containing amino acids, cysteine and methionine. The functional significance of this observation is unknown, but it may be of some practical interest in further work with this protein.

The data (presented in Fig. 8B) relating to the kinetics and stoichiometry of interaction of P11 and 11- defective bacteriophage particles present some problems in interpretation. That at relatively low P11 concentrations the maximum rate of activation and end point titers manifest a similar P11 concentration dependence (formally, 1,5 order) is a satisfying internal consistency. The end point titer curve shows a sharp break at the point which represents an equivalence of P11 and 11- defective bacteriophage particles. The maximum rate of activation curve continues to rise, however, with approximately the same order of concentration dependence to a P11 concentration several times equivalency, where there is a marked change toward Pll concen-

tration dependence to a Pll concentration several times equivalency. The end point titer curve shows a sharp break at the point which represents an equivalence of P11 and 11- defective bacteriophage particles. The maximum rate of activation curve continues to rise, however, with approximately the same order of concentration dependence to a P11 concentration several times equivalency, where there is a marked change toward P11 concentration dependence. This probably indicates that another component of the system is becoming rate-limiting. P12, which is known to attach obligatorily to the bacteriophage particle after Pll is biologically active, yet physically indistinguishable (by the limited criteria employed in this investigation) from the bulk inactive form. The observation (7) that P11 interacts with P10 during the course of bacteriophage development may be of some relevance, since, in the isolation reported here, the starting material was a 10- defective lysate. Although, as of this writing, this has not been investigated further, it is anticipated that P11 will be isolated from a defective lysate other than 10-, as well as from complete bacteriophage particles. Examination of the chemical and biological properties of P11 isolated from these alternate sources may be expected to shed some light on the high equivalence number observed in the experiments reported in this communication. It is anticipated further that such experiments shall yield information concerning changes in protein structure upon incorporation of the nascent polypeptide chain into the bacteriophage structure.

The assay system for P11 described in this report was necessarily crude and qualitative. As shown by an investigation of optimal pH and ionic concentration, 0.1 m phosphate buffer at pH 6.2 would have been slightly preferable to the 0.07 m phosphate buffer at pH 7.5 actually used. Furthermore, the over night (12- to 18-hour) incubation was not necessarily sufficiently long to give an end point titer of activated bacteriophage, particularly at the more dilute end of the range. However, even as performed, there was sufficient internal consistency to make this a valid and valuable qualitative assay for the purposes of identification of P11 in the separation procedures. That the assay as performed manifested a second order P11 concentration dependence (which was used in recovery and purification calculations), compared with the 15th order observed in the careful end point titrations, is attributable to the relatively short term incubations performed in the former case.

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