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Purification and Properties of the σ Subunit of *Escherichia coli* DNA-Dependent RNA Polymerase†

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ABSTRACT: An improved purification procedure is described for the σ subunit of *Escherichia coli* DNA-dependent RNA polymerase [ribonucleoside triphosphate:RNA nucleotidyltransferase, EC 2.7.7.6]. The method involves chromatography of purified RNA polymerase on single-stranded DNA–agarose, Bio-Rex 70, and finally Ultragel AcA44. The σ factor obtained is electrophoretically pure with a yield of about 40%. A number of the chemical–physical properties of σ are presented. A molecular weight of 82 000 was determined by phosphate buffered sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Ultraviolet absorption spectra were used to determine an $E_{280nm}^{1\%}$ of 8.4. The amino acid composition and 12-residue N-terminal sequence (Met-Glx-Glx-Asx-Pro-Glx-(Ser or Cys)-Glx-Leu-Lys-Leu-Leu) of σ have been determined. The isoelectric focusing properties of σ are presented. Denaturation–renaturation studies indicate that σ is capable of an unusually rapid and complete recovery of activity after being subjected to denaturing conditions. A stable, 40 000-dalton fragment is generated from σ by mild trypsin treatment.

Escherichia coli DNA-dependent RNA polymerase holoenzyme ($\alpha_2\beta\beta'\sigma$) can be reversibly dissociated into a σ subunit and a core polymerase ($\alpha_2\beta\beta'$) (Burgess et al., 1969; Burgess, 1971). The σ subunit is used catalytically and is required for efficient polymerase binding and initiation of RNA synthesis at bacterial and bacteriophage promoters (Travers & Burgess, 1969; Chamberlin, 1976). Recently the location of the σ gene has been determined to be at about 66 min of the *E. coli* genetic map. Nakamura et al. (1977) and Harris et al. (1977) showed that a locus affecting the size of σ mapped in this region. Mutants affecting σ activity map in this region (Gross et al., 1978; Travers et al., 1978; Nakamura, 1978; Harris et al., 1978). Finally, three different mutants of σ have been shown to differ from wild type σ and each other in their tryptic peptide patterns (Burgess, R., Gross, C., and Lowe, P., unpublished observations).

Although several papers dealing with the purification of σ have appeared (Burgess et al., 1969; Burgess & Travers, 1971; Berg et al., 1971; Burgess, 1976), detailed studies of the chemical and physical properties of σ and its role in transcription have been delayed by the difficulty of obtaining milligram amounts of σ with satisfactory purity. We present here the details of a method for obtaining pure σ in amounts to allow its physical and chemical characterization. The purification procedure uses as starting material the RNA polymerase purified by the method of Burgess & Jendrisak (1975) through the Polymin P precipitation, DNA cellulose, and gel filtration chromatography steps. This RNA polymerase does contain minor impurities and is not saturated with σ or free of subassemblies caused by polymerase dissociation. Therefore, we purify it further and resolve it into core polymerase and holoenzyme by stepwise elution from a single-stranded DNA–agarose (Nüsslein & Heyden, 1972). Pure holoenzyme is separated into core polymerase and σ by chromatography on a tandem column of Bio-Rex 70 and DEAE-cellulose. Final purification of σ is achieved by gel filtration chromatography on an Ultragel AcA44 column. The purification scheme presented also includes details for producing highly purified holoenzyme and core polymerase.

We present a number of the chemical and physical properties of σ, including molecular weight, extinction coefficient, absorption spectrum, amino acid analysis, N-terminal sequence, isoelectric focusing characteristics, denaturation–renaturation properties, and proteolytic fragmentation pattern.

Materials and Methods

A. Materials. Guanidine hydrochloride was purchased from Schwarz/Mann and phenylmethanesulfonyl fluoride from Calbiochem. Electrophoresis grade acrylamide, sodium dodecyl sulfate, agarose, *N,N'*-methylenebisacrylamide, *N,N,N',N'*-tetramethylethylenediamine, and ammonium persulfate were obtained from Bio-Rad. Nonidet-P40 was purchased from Gallard-Schlessinger. Urea (technical grade) from Baker was used without further purification. Purified rabbit muscle myosin was a gift from M. Greaser. *E. coli* β-galactosidase, ovalbumin, trypsin, and highly polymerized calf thymus DNA were obtained from Worthington. Bovine serum albumin was purchased from Miles. Beef liver catalase and rabbit muscle phophorylase *a* were obtained from Sigma.

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Soluene-100 was purchased from Packard. ATP, UTP, CTP, and GTP were obtained from P-L. Biochemicals. [^{3}H]UTP was purchased from the Radiochemical Centre, Amersham. T4 DNA was prepared as described by Gross et al. (1976).

B. Buffers. Buffers and stock solutions for the purification of σ were the same as those used for the purification of holoenzyme (Burgess & Jendrisak, 1975). All pH values quoted were determined at 20 °C. The basic buffer throughout was TGED:[1] 0.01 M Tris-HCl, pH 7.9, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 5% glycerol, containing NaCl at the concentration noted. Storage buffer for σ was TGED + 0.5 M NaCl with 50% (v/v) glycerol instead of 5%. Normal storage buffer (TGED + 0.1 M NaCl and 50% glycerol) was used for holoenzyme and core polymerase.

C. Chromatography. Single-stranded calf thymus DNA–agarose was prepared as described by Schaller et al. (1972) and Nüsslein & Heyden (1972) with the following modifications: calf thymus DNA was thoroughly dissolved in 0.02 M NaOH to a concentration of 12 mg/mL. The homogeneous solution was heated to 50 °C and mixed with a half volume of 6% (w/v) agarose which had been autoclaved to dissolve the agarose and cooled to 50 °C. The solution was mixed thoroughly for 5 min at 50 °C in a large shallow dish and then allowed to gel on ice. The gel was sliced into approximately 1-cm cubes and immersed in 1 M Tris-HCl, pH 7.9, before passing twice through a stainless steel mesh. A column was filled with the resulting material and washed with TGED + 1 M NaCl, followed by at least 5 column volumes of TGED + 0.25 M NaCl. About 80% of the DNA was recovered as single-stranded DNA–agarose (approximately 4 mg of DNA/mL of packed column). Bio-Rex 70 (Bio-Rad) was precycled in NaOH/HCl as described previously (Burgess & Jendrisak, 1975). Ultragel AcA44 (LKB) was suspended in its equilibration buffer and packed into a column.

D. Protein Determinations. Protein concentrations were determined spectrophotometrically using $E_{280nm}^{1\%}$ values of 6.2 for holoenzyme, 5.5 for core enzyme (Burgess, 1976), and 8.4 for σ (see present work). Measurements were made in 1-mL, 1-cm path length cuvettes on a Beckman Acta III spectrophotometer in the double-beam mode and standardized against a solution of 0.050 g of potassium dichromate in 1 L of 0.01 N H_2SO_4 (Rand, 1969). The $E_{280nm}^{1\%}$ of σ was measured by the method of Scopes (1974) and its tryptophan content determined as described by Edelhoch (1967). σ protein measurements on a microscale were carried out by the method of Schaffner & Weissmann (1973) using calibration curves of bovine serum albumin and σ. Ten micrograms of bovine serum albumin bound 0.86 times the amido black dye of 10 μg of σ [weights of bovine serum albumin and σ were determined from their extinction coefficients at 280 nm using values of 6.6 (Edsall & Spahr, 1962) and 8.4, respectively]. The σ content, or saturation, of holoenzyme was determined by analyzing a sample by Tris–glycine sodium dodecyl sulfate–polyacrylamide gel electrophoresis, staining with Coomassie Brilliant Blue, destaining, and scanning the gel at 550 nm (Burgess, 1976). The area under a scan of σ was compared with that of α and saturation calculated using a σ molecular weight of 82000 and two α polypeptides of molecular weight 36512 and assuming σ and α bind the same amount of dye per microgram. The purity of σ was determined by scanning destained gels as above. Gel scans were performed on the above spectrophotometer fitted with a scanning attachment.

[1] Abbreviation used: TGED, 0.01 M Tris-HCl (pH 7.9), 0.1 mM EDTA, 0.1 mM dithiothreitol, and 5% glycerol.

E. Activity Assays. The assay mixture (in 100 μL) was 0.04 M Tris-HCl, pH 7.9, 0.2 M NaCl, 0.01 M $MgCl_2$, 1 mM EDTA, 0.2 mM CTP, GTP, and ATP, 0.05 mM UTP, 1 μCi of [^{3}H]UTP, 3.5 μg of T4 DNA, 1 mM K_2HPO_4, 1 mM dithiothreitol, and 50 μg of bovine serum albumin (Gross et al., 1978). After incubation for 10 min at 37 °C, 20 μL of 2.5% sodium dodecyl sulfate, 0.15 M sodium pyrophosphate was added, and 100 μL of the assay mixture was applied directly to DEAE-cellulose (Whatman DE 81) disks. The disks were washed by soaking in 5% Na_2HPO_4, water, ethanol, and ether as described by Somers & Pearson (1975). The dried disks were placed in scintillation vials and the label was solubilized in 0.5 mL of 95% Soluene-100 for 1 h at room temperature prior to liquid scintillation counting in a toluene-based counting fluid (Dyan et al., 1977).

σ activity was measured by its ability to stimulate transcription of T4 DNA in assays supplemented with 1 μg of core polymerase. Incorporation of label from [^{3}H]UTP increased linearly with increasing σ concentration up to approximately 0.05 μg of σ. Maximum stimulation of core polymerase was 40–60-fold, reaching the specific activity given by an equivalent amount of holoenzyme. σ activity is defined as follows: 1 unit of σ activity produces a half-maximum stimulation of transcription on T4 DNA by 1 μg of core polymerase in the standard assay. This corresponds to approximately 0.02 μg of electrophoretically pure σ.

F. Polyacrylamide Gel Electrophoresis. Stacking Tris–glycine sodium dodecyl sulfate–polyacrylamide gels (Laemmli, 1970) were run as described by Burgess & Jendrisak (1975). Nonstacking phosphate buffered sodium dodecyl sulfate gels were prepared by the method of Weber & Osborn (1975).

G. Amino Acid Analysis. One milligram of σ was dialyzed against 0.1 M NaCl, followed by exhaustive dialysis against double-distilled water. Precipitated σ was transferred into several thick-walled Pyrex boiling tubes and lyophilized, and the samples were hydrolyzed for 24, 48, or 76 h in 6 N HCl containing 0.1% double-distilled phenol and 1% thiodiglycolic acid in a boiling toluene bath at 110 °C as described by Moore & Stein (1963). Performic acid oxidation was carried out by the method of Moore (1963). Samples were chromatographed in duplicate on a Durrum amino acid analyzer.

H. N-Terminal Sequence. Identification of the 12 N-terminal amino acid residues of σ was carried out by the quantitative Edman-Begg Sequenator procedure of Smithies et al. (1971). Two milligrams of σ was loaded onto a Illitron sequenator. Both acid hydrolysis with HI and basic hydrolysis with NaOH–$Na_2S_2O_4$ were used to regenerate parent amino acids from their thiazolinones.

I. Isoelectric Focusing. First-dimension isoelectric focusing was carried out by a modified version of the O'Farrell (1975) procedure. For most of the studies a modified anode reservoir buffer was used, consisting of 8 M urea, 2% Nonidet-P40 in degassed water, adjusted to pH 2.5 with 18 mL of 85% phosphoric acid per liter. This resulted in a sharper σ band. Focusing was performed at 350 V for 18 h, followed by 1 h at 800 V in 130 × 2.5 mm i.d. tubes. Gels were stained in 0.05% Coomassie Brilliant Blue R-250 in 95% ethanol–acetic acid–water (5:1:5) for 1 h at 60 °C and destained by soaking overnight in 15 mL of the above solvents (2.5:1:6.5), followed by storage in 7.5% acetic acid. Prolonged destaining in several changes of 95% ethanol–acetic acid–water (2.5:1:6.5) causes the protein bands to fade, whereas soaking in 7.5% acetic acid alone fails to destain effectively. The pH gradient of the first dimension was determined by slicing the gel into 1-cm sections and shaking each slice for 3 h with 2 mL of degassed dou-

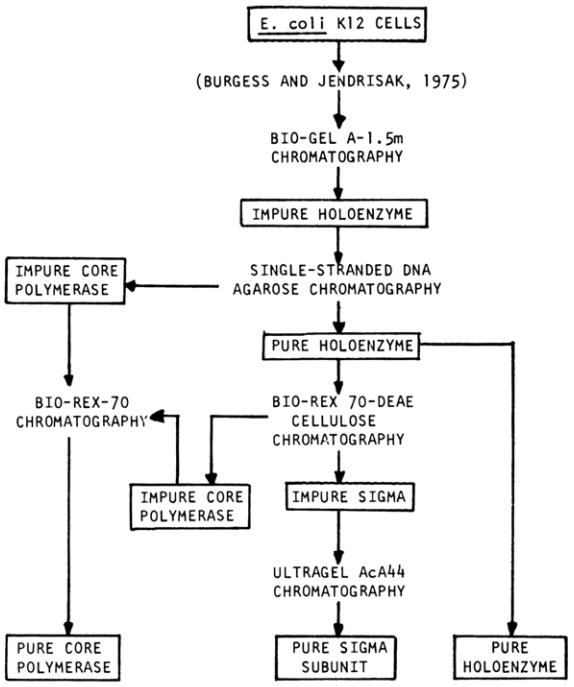

FIGURE 1: Schematic for the isolation of pure σ subunit, holoenzyme, and core polymerase.

ble-distilled water in tightly capped tubes. Second-dimension, Tris–glycine sodium dodecyl sulfate–8.75% polyacrylamide slab gels were run as described by O'Farrell (1975).

Purification Procedure

A schematic for the purification procedure is given in Figure 1. All steps were carried out at 4 °C.

A. RNA Polymerase Starting Material. RNA polymerase used as starting material for the purification of σ was isolated from *E. coli* K12 cells (500 g) as described by Burgess & Jendrisak (1975). Several improvements to that procedure are discussed later. In particular, chromatography on Bio-Gel A-5m (the final step in the purification of the enzyme by that method) has been modified by substitution of an identical column of Bio-Gel A-1.5m.

B. Single-Stranded DNA–Agarose Chromatography. The first two-thirds of the RNA polymerase peak eluting from the Bio-Gel A-1.5m column (about 110 mg of protein) was pooled, diluted with TGED to TGED + 0.25 M NaCl and applied, at 40 mL/h, to a 25-mL (1.8 × 10 cm) single-stranded DNA–agarose column equilibrated with TGED + 0.25 M NaCl. After washing with 40 mL of TGED + 0.25 M NaCl, core polymerase was eluted with 50 mL of TGED + 0.4 M NaCl. Holoenzyme was eluted with TGED + 1 M NaCl at 20 mL/h (Figure 2). Core polymerase and holoenzyme were pooled separately.

C. Bio-Rex 70/DEAE-cellulose Chromatography. A 45-mL (1.5 × 25 cm) Bio-Rex 70 column with its outflow connected to a 3-mL (1 × 3.9 cm) DEAE-cellulose column was equilibrated with TGED + 0.1 M NaCl. Pooled holoenzyme (a sample of 56 mg from the previous step) was diluted with TGED to 0.1 M NaCl, applied to the columns at 50 mL/h, and washed with 100 mL of TGED + 0.1 M NaCl. The core polymerase binds to Bio-Rex 70. The released σ flows through and is bound by the DEAE-cellulose column. The DEAE-cellulose column was disconnected from the Bio-Rex 70 column and washed with a further 10 mL of buffer, followed by elution of σ with TGED + 0.5 M NaCl at 5 mL/h. Core polymerase was eluted from the Bio-Rex 70 column with a 300-mL gradient from TGED + 0.1 M NaCl to TGED + 0.5 M NaCl. The trace amounts of σ that remain bound to core polymerase may be removed by diluting the pooled core fractions with TGED to 0.1 M NaCl and repeating the Bio-Rex 70 chromatography.

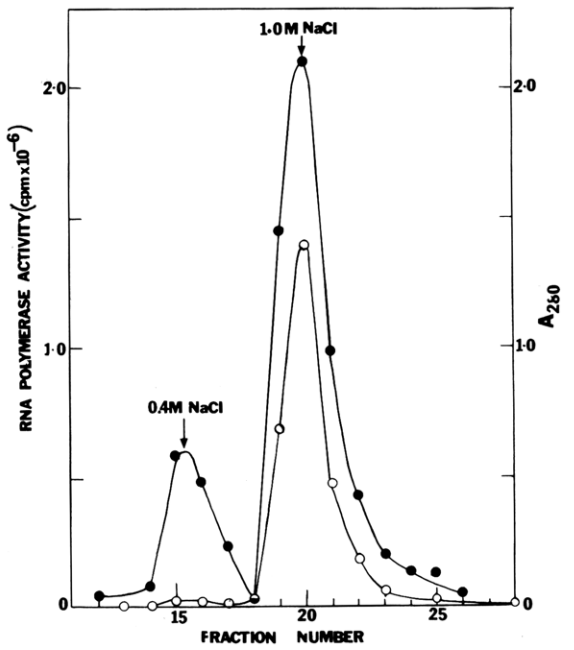

FIGURE 2: Chromatography of RNA polymerase on single-stranded DNA–agarose. Purified enzyme (110 mg) from the Bio-Gel A-1.5m column was chromatographed as described in the Purification Procedure section. Fractions 14–17 (pooled core polymerase) and 19–22 (pooled holoenzyme) were 12 and 7 mL each, respectively. Fractions 23–27 were holoenzyme side fractions. Five-microliter samples were removed and diluted appropriately in TGED (where necessary) prior to assay for RNA polymerase activity. (●) Absorbance at 280 nm. (○) Activity per 5-μL sample measured on T4 DNA; 80 440 cpm is equivalent to 1 nmol of [^{3}H]UMP incorporated into RNA.

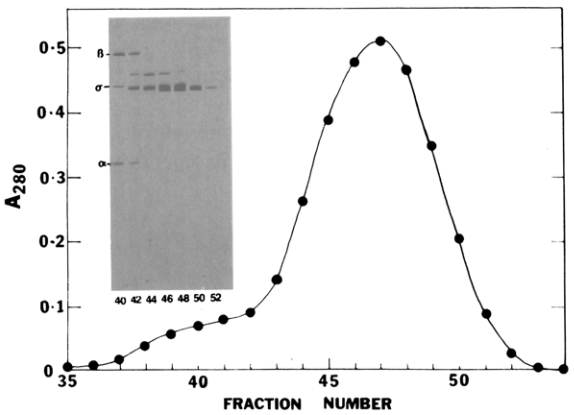

FIGURE 3: Chromatography of partially purified σ on Ultragel AcA44. Impure σ (4.5 mg), eluted stepwise from the DEAE-cellulose column, was chromatographed on Ultragel AcA44 as described in the Purification Procedure section. One-milliliter fractions were collected and their absorbance read at 280 nm (●). The inset shows Tris–glycine sodium dodecyl sulfate–10% polyacrylamide gel analysis of Ultragel AcA44 column fractions. Twenty microliters of fractions 40 and 42 and 10 μL of fractions 44, 46, 48, 50, and 52 were analyzed.

D. Ultragel AcA44 Chromatography. Pooled σ containing fractions (4.5 mL) from the DEAE-cellulose column were applied to a 120-mL (1.5 × 68 cm) column of Ultragel AcA44 equilibrated with TGED + 0.5 M NaCl. The column was developed at 5 mL/h with TGED + 0.5 M NaCl, and 1.0-mL fractions were collected (Figure 3). Samples of appropriate fractions across the complete protein profile were analyzed on Tris–glycine sodium dodecyl sulfate–10% polyacrylamide gels

(see inset, Figure 3). Fractions containing σ free of $\alpha_2\beta$ were pooled, dialyzed against 1000 mL of storage buffer, aliquoted, and stored at −20 or −70 °C.

Comments on the Purification Steps

A. RNA Polymerase Starting Material. The cells used were obtained from Grain Purchasing Corp. (grown on enriched medium to one-half log phase), but similar results have been obtained with cells we have grown ourselves. Several corrections and improvements in the procedure of Burgess & Jendrisak (1975) should be noted.

After completely resuspending the pieces of frozen *E. coli* in grinding buffer, the blender was placed in a 20 °C water bath and the mixture stirred with a glass rod until a temperature of around 5 °C was reached. The suspension was maintained at this temperature for 30 min prior to the addition of sodium deoxycholate. This increased temperature allows more effective lysozyme treatment and is needed for good lysis with many strains of *E. coli*. The DNA shearing procedure was modified to minimize foaming and thus facilitate centrifugation. The DNA shearing of the detergent-treated cells was accomplished by doubling the time of high-speed blending to 60 s. After addition of 2000 mL of TGED + 0.2 M NaCl, the extract was blended for 30 s at low, not high, speed, to achieve a homogeneous mixture and to minimize foaming just prior to centrifugation.

DNA-cellulose was prepared as before except that calf thymus DNA at 6 mg/mL, instead of at 2 mg/mL, was used, resulting in column material containing about 1.5 mg of DNA/mL of packed column. This allows the use of a smaller column (150 mL) with its associated better flow rate and resolution. We now elute this column with a salt gradient from TGED + 0.15 M NaCl to TGED + 0.5 M NaCl.

As mentioned earlier, we have replaced the Bio-Gel A-5m column with an identically sized column of A-1.5m. This allows the RNA polymerase to elute closer to the void volume and thus farther from the lower molecular weight impurities, including several ribonucleases. LKB Ultragel AcA22 has also been used successfully for this purpose. We recommend that samples of appropriate fractions from the Bio-Gel A-1.5m profile be analyzed on Tris–glycine sodium dodecyl sulfate–10% polyacrylamide gels prior to pooling for σ purification. Only enzyme from the first two-thirds to three-fourths of the peak should be pooled because the last one-third to one-fourth contains very little σ and occasionally contaminants which subsequently copurify with σ. Yields of approximately 150 mg of polymerase from the A1.5m column are more typical than the occasional high yield of almost 250 mg reported earlier (Burgess & Jendrisak, 1975). A detailed discussion of many aspects of the purification procedure has been presented elsewhere (Burgess, 1976).

B. Single-Stranded DNA–Agarose Chromatography. Single-stranded DNA–agarose chromatography provides a very convenient and powerful purification step (Schaller et al., 1972; Nüsslein & Heyden, 1972). Some impurities and $\alpha_2\beta$ subassembly flow through the column at 0.25 M NaCl. We have found step elution much more satisfactory than the gradient elution of Nüsslein & Heyden (1972) since the enzymes elute in a much more concentrated form. The optimal salt concentrations used for the elution of core polymerase and holoenzyme were previously determined in the study of deHaseth et al. (1978). At 0.4 M NaCl core polymerases elutes rapidly, but there is a very slow constant rate of holoenzyme elution. Therefore the 0.4 M NaCl elution cannot be continued much longer than suggested without decreasing the recovery of holoenzyme in the 1 M NaCl step. This core

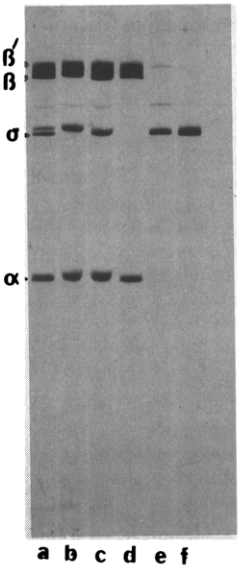

FIGURE 4: Tris–glycine sodium dodecyl sulfate 10% polyacrylamide gels of various fractions of the purification: (a) 10 μg of holoenzyme from the Bio-Gel A-1.5m column (used as starting material for the purification of σ; (b) 10 μg of core polymerase eluted from the single-stranded DNA–agarose column by TGED + 0.4 M NaCl (N.B. the major band migrating between ββ′ and α is an impurity with a slightly higher molecular weight than σ); (c) 10 μg of pure holoenzyme eluted from the single-stranded DNA–agarose column by TGED + 1 M NaCl; (d) 8 μg of core polymerase eluted from the Bio-Rex 70 column; (e) 2 μg of partially purified σ eluted from the DEAE-cellulose column; (f) purified σ, 3 μg of fraction 50 from the Ultragel AcA44 column.

polymerase (Figure 4, gel b) must be rechromatographed on Bio-Rex 70 to remove the impurity mentioned below and traces of σ. The flow rate is decreased during the 1 M NaCl elution to allow more time for the holoenzyme to dissociate from the column and to increase the concentration of protein in the fractions. The high DNA content of the column and the high RNA polymerase binding capacity of single-stranded DNA allow one to apply up to 100 mg of polymerase to a 20–30-mL column.

A band occasionally present in our polymerase preparations has a mobility slightly less than σ on sodium dodecyl sulfate gels. This protein, which elutes just before and with the core polymerase peak, can be seen in Figure 4, gels a and b. It is identical with the σ-sized protein eluting with core polymerase observed by Nüsslein & Heyden (1972) but is apparently unrelated to RNA polymerase or σ. Highly purified holoenzyme, used routinely for transcription studies in our laboratory, is obtained by dialyzing the pooled holoenzyme peak against a storage buffer (TGED + 0.1 M NaCl + 50% glycerol) and storing at −70 °C (see Figure 4, gel c).

C. Bio-Rex 70/DEAE-cellulose Chromatography. The reasons for the superiority of Bio-Rex 70 chromatography over phosphocellulose for the separation of σ and core polymerase have been discussed elsewhere (Burgess, 1976). Concentration of dilute σ on a small column of DEAE-cellulose was originally described by Berg et al. (1971). Stepwise elution of σ from the 3-mL DEAE-cellulose column gives a sufficiently small sample volume to allow direct application to the Ultragel AcA44 column without further concentration. To obtain high-quality core polymerase, as free as possible of residual σ, the two cycles of Bio-Rex 70 chromatography are necessary. It is also important, when pooling fractions from the first Bio-Rex 70 column, to discard the leading 10% of the core polymerase peak since it seems to contain most of the remaining σ. The final pooled core polymerase is dialyzed

Table I: Summary of Purification[a]

fraction	vol (mL)	total protein (mg)[c]	total σ protein (mg)	σ yield[b] (%)	σ act. (units)	σ sp act. (units/mg)
Bio-Gel pooled peak	104	110 (44%)[e]	8.35[b]	100		
single-stranded DNA–agarose column						
pooled core polymerase (fractions 14–17)	48	24.2				
pooled holoenzyme peak (fractions 19–22)	28	56.3 (59%)[e]	5.73[b]	78		
holoenzyme peak side fractions (23–26)[d]	29	6.3 (68%)[e]	0.74[b]			
Bio-Rex 70 DEAE-cellulose column						
σ from DEAE-cellulose column	4	4.53	3.85 (85%)[f]	52	138 600	30 600
DEAE-cellulose side fractions[d]	3	0.49	0.42 (85%)[f]			
core polymerase	60	51				
Ultragel AcA44 column pooled fractions						
40–43	4	0.61	0.24 (40%)[f]		6 700	11 000
44–46	3	1.36	1.20 (88%)[f]	37	37 900	27 900
47–51	5	1.97	1.91 (97%)[f]		58 500	29 700
			3.35 (total)			

[a] From 500 g of cells. [b] Determined by quantitation of σ on gels (Burgess, 1976). [c] Determined spectrophotometrically using the appropriate extinction coefficients. [d] These fractions were not used in subsequent σ purification. [e] σ saturation in parentheses. [f] Purity of σ in parentheses determined as in *b*.

against storage buffer and stored at −70 °C (see Figure 4, gel d).

D. Ultragel AcA44 Chromatography. Although Ultragel AcA44 does not have the resolving power of zonal centrifugation for the purification of σ, it effectively separates σ from $\alpha_2\beta$. The chromatographic procedure is more convenient than centrifugation and has a higher sample volume capacity, and the column can be used repeatedly and highly reproducibly. This obviates the need for multiple centrifugations or further concentration of σ.

E. Purity and Yield. Fractions from various stages of the purification have been analyzed by gel electrophoresis (Figure 4) and have been assayed for protein content and enzymatic activity (Table I).

The final purified σ (yield about 40%) is usually split into three batches (Table I). σ of highest electrophoretic purity (97%) is used for protein chemistry studies such as those presented later in this paper and precise σ activity studies. σ of intermediate purity (88%) contains an impurity band, discussed below. This batch of σ is used for routine activity determinations since we have no evidence that the impurity interferes with the assaying of σ. The small amounts of σ of low purity (40%), containing mainly $\alpha_2\beta$, may be rechromatographed or used for preliminary activity studies.

The major contaminant present in σ of intermediate purity is a protein with a molecular weight of about 110 000. This protein comigrates on 10% polyacrylamide Tris–glycine sodium dodecyl sulfate gels with the large fragment generated by partial proteolytic digestion of β′ (Lowe & Malcolm, 1976). We suspect that this contaminant is a clipped form of β′ which remains bound to RNA polymerase throughout the purification and is removed, as is σ, by Bio-Rex 70 chromatography. Since the impurity is slightly larger than σ, it is only partially resolved from σ by AcA44 chromatography. We have not yet found a satisfactory way of separating the contaminant from σ. The amount varies considerably. Sometimes the impurity is not detectable; at other times it is even more abundant than in the preparation presented here (see note added in proof).

The in vivo level of σ in *E. coli* has been estimated at 30–40% saturation (0.3–0.4 molecules of σ per molecule of core polymerase, discussed by Burgess, 1976). σ levels in the Bio-Gel A-1.5m product vary from 40 to 80% saturation. This appears to vary with strain, harvesting point on the growth curve, and media, but no consistent picture has emerged. The σ saturation of pooled holoenzyme from the single-stranded DNA–agarose column was between 60 and 70% in this preparation. However, saturation levels of greater than 90% are often obtained. It is not yet understood why some core polymerase is present in the holoenzyme peak of this column, but perhaps it is due to core polymerase trailing into the holoenzyme peak.

The yield of σ with highest and intermediate purity is 37% of the σ present in the RNA polymerase applied to the single-stranded DNA–agarose column (Table I). A somewhat higher yield may be obtained by wider pooling of fractions at intermediate steps of the purification. The overall yield of σ is probably less than 10% of the σ present in the cells. We find that significant losses of σ occur if fractions from the last three columns are not collected in plastic tubes, presumably due to σ adhering to glass tubes. Essentially no loss of protein or activity occurs during dialysis of the final product into storage buffer.

Physical–Chemical Properties

A. Molecular Weight. The comparative mobility of σ with a series of standard proteins on Tris–glycine sodium dodecyl sulfate gels and phosphate-buffered sodium dodecyl sulfate–polyacrylamide gels is shown in Figure 5. σ migrates closer to the phosphorylase marker in the Tris–glycine gel system than in the phosphate gel system. Standard proteins on both gel systems generate linear plots of mobility vs. log molecular weight. We obtain apparent molecular weights for σ of 90 000 on Tris–glycine sodium dodecyl sulfate gels and 82 000 on phosphate-buffered sodium dodecyl sulfate gels.

Mobility discrepancies have been reported for a number of proteins analyzed on the two gel systems (Swaney et al., 1973;

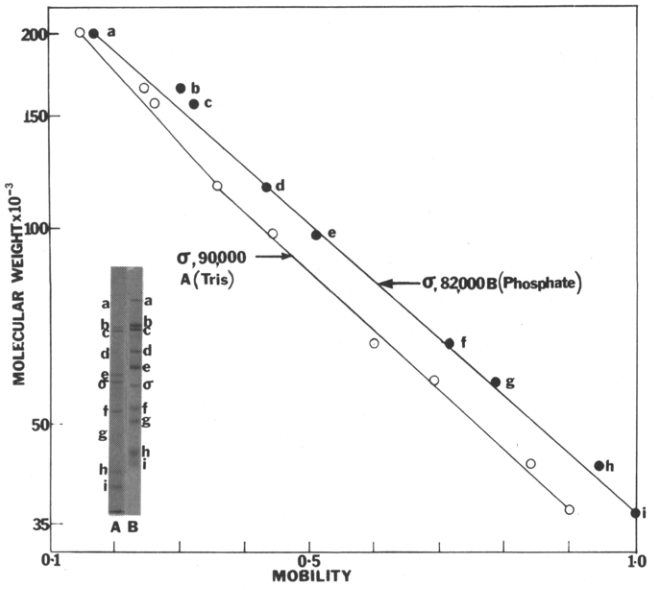

FIGURE 5: Molecular weight determination of *E. coli* RNA polymerase σ subunit. A plot of mobility vs. molecular weight for σ and marker proteins on Tris–glycine sodium dodecyl sulfate–7.5% polyacrylamide gels (○) and phosphate-buffered sodium dodecyl sulfate–5% polyacrylamide gels (●). The marker proteins were (a) myosin, molecular weight 200 000 (Weber & Osborn, 1975); (b) β′ and β subunits of *E. coli* RNA polymerase, molecular weights 165 000 and 155 000, respectively (Burgess, 1969); (c) β-galactosidase, molecular weight 116 248 (Fowler & Zabin, 1977); (e) phosphorylase, molecular weight 97 412 (Titani et al., 1977); (f) bovine serum albumin, molecular weight 66 296 (Brown, 1976); (g) catalase, molecular weight 58 000 (Weber & Osborn, 1975); (h) ovalbumin, molecular weight 43 000 (Castellino & Barker, 1968); and (i) the α subunit of *E. coli* RNA polymerase, molecular weight 36 512 (Ovchinnokov et al., 1977). Gels were run in batches of nine and each run was repeated in triplicate for both gel systems. Mobilities are relative to bromophenol blue tracker dye in the case of Tris–glycine sodium dodecyl sulfate gels and to the α subunits in the case of phosphate-buffered sodium dodecyl sulfate gels. The inset shows migration of σ and marker proteins on Tris–glycine sodium dodecyl sulfate–7.5% polyacrylamide gels (A) and phosphate-buffered sodium dodecyl sulfate–5% polyacrylamide gels (B).

Camacho et al., 1975). The Tris–glycine sodium dodecyl sulfate gel system appears to be susceptible to the generation of anomalous mobilities whereas the phosphate-buffered sodium dodecyl sulfate gel system is not. We have, therefore, used a σ molecular weight of 82 000 for subsequent calculations. A molecular weight of 82 000 is smaller than the σ molecular weights published to date (85 000, Zillig et al., 1970b; 87 000, Berg et al., 1971; 95 000, Burgess, 1969). In this work, the molecular weights of some of the marker proteins were taken from complete amino acid sequences and are significantly lower than had been previously supposed. It should be noted that β′ and β both fall above the calibration line in Figure 5 when plotted using the molecular weights 165 000 and 155 000, respectively (Burgess, 1969). Since these earlier values were obtained using marker proteins with estimated molecular weights higher than the recently obtained sequence molecular weights, it is likely that β′ and β are smaller than originally thought, closer to the values of 150 000 and 145 000, respectively, reported by Berg et al. (1971). More definitive values are difficult to obtain due to the paucity of well-characterized high-molecular-weight markers.

B. Spectrophotometric Properties. The ultraviolet absorbance spectrum of electrophoretically pure σ is given in Figure 6. The protein has an A_{280}/A_{260} ratio of 1.8, indicating no detectable contamination with nucleic acid. Appropriate

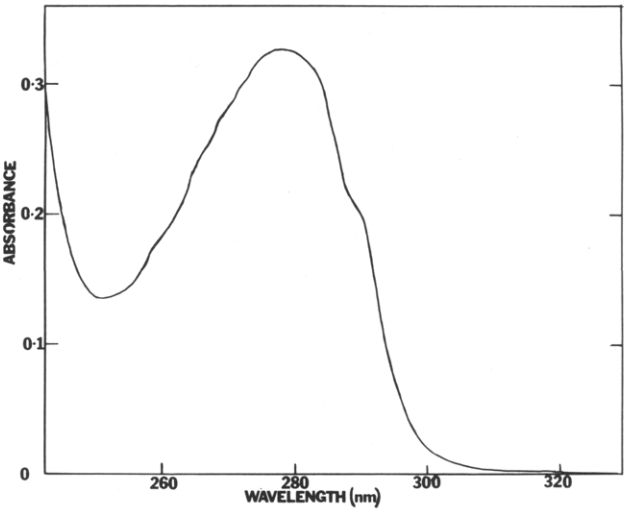

FIGURE 6: Absorbance spectrum of 0.38 mg/mL solution of electrophoretically pure σ in 0.1 M K_2SO_4, 5 mM KH_2PO_4, pH 7.0. This spectrum and the absorption at 205 nm of a dilution of this solution was used to calculate an $E_{280nm}^{1\%} = 8.4$.

dilutions of this sample were used to obtain the spectrum between 190 and 220 nm. The value for A_{280}/A_{205} (0.0276) was substituted in the following equation: $E_{205nm}^{0.1\%} = 27 + 120 \times (A_{280}/A_{205})$ (Scopes, 1974). Using this value for the extinction coefficient at 205 nm, the absolute concentration of σ was obtained and an $E_{280nm}^{1\%}$ of 8.4 calculated. This represents a slight downward revision of the value of 8.8 reported earlier (Burgess, 1976). No correction for light scattering was necessary.

Absorption spectra of σ in 6.5 M guanidine hydrochloride (pH 6.5) were used to calculate the number of tryptophan residues (N_{Trp}) using the equation: $N_{Trp} = E'_{288nm}/3103 - E'_{280nm}/10318$ (Edelhoch, 1967), where E' is the molar extinction coefficient. A total of eight tryptophan residues per σ molecule was obtained.

C. Amino Acid Composition. The amino acid composition of σ is presented in Table II. As expected from its markedly acidic behavior on various gel systems (urea–polyacrylamide, Burgess et al., 1969; cellulose acetate–urea, Zillig et al., 1970a; and isoelectric focusing gels, see present work), the amount of Glx and Asx is high compared with that found in the other subunits of RNA polymerase (Burgess, 1976). Another feature is the relatively low level of cysteine/cystine residues.

The σ amino acid composition presented here differs in many residues from that obtained by Fujiki & Zurek (1975).

D. N-Terminal Sequence. The following four-residue N-terminal sequence for σ has been determined by Fujiki & Zurek (1975) by micro-Edman degradation:

$$\underset{1\quad 2\quad 3\quad 4}{H_2N\text{-Met-Gly-Gln-Asn-COOH}}$$

We have obtained the following 12-residue N-terminal sequence by the quantitative sequenator method of Smithies et al. (1971):

$$\underset{1\quad 2\quad 3\quad 4\quad 5\quad 6}{H_2N\text{-Met-Glx-Glx-Asx-Pro-Glx-}}$$

$$\underset{7\qquad 8\quad 9\quad 10\quad 11\quad 12}{\text{(Ser or Cys)-Glx-Leu-Lys-Leu-Leu-COOH}}$$

Ser and Cys are indistinguishable using this method. The latter sequence differs from the former in having Glx (either Glu or Gln) in position 2 instead of Gly. This discrepancy

Table II: Amino Acid Composition of σ

amino acid	mol %[a]	residues/σ molecule[f]
Asx	11.46 (±0.07)	83
Thr[b]	7.19 (±0.15)	52
Ser[b]	5.07 (±0.1)	37
Glx	15.26 (±0.23)	110
Pro	3.14 (±0.11)	23
Cys[c]	0.61	4
Gly	4.17 (±0.03)	30
Ala	7.88 (±0.04)	57
Val[d]	5.64 (±0.12)	41
Met[g]	4.00	(29)[g]
Ile[d]	6.90 (±0.14)	50
Leu	9.04 (±0.12)	65
Tyr	1.85 (±0.18)	13
Phe	2.49 (±0.04)	18
Lys	6.01 (±0.12)	43
His	1.66 (±0.03)	12
Arg	6.52 (±0.29)	47
Trp[e]	1.11	8
	total	722

[a] Average of three determinations. Standard error of the mean at a confidence level of $p = 0.05$ is indicated. [b] Extrapolated to zero time from determinations at 24, 48, and 72 h of hydrolysis. [c] Too small to measure accurately. The value quoted is recalculated from the data (Burgess et al., 1976) using the value for Leu in this paper and the sequence for β-galactosidase of Fowler & Zabin (1977). [d] Values reached after 72 h of hydrolysis. [e] Determined spectrophotometrically by the method of Edelhoch (1967). [f] Calculated using a mean residue molecular weight of 114 and a σ molecular weight of 82 000. [g] Determined as methionine sulfone. Other reported values for mole percent Met are 2.81, calculated as for Cys in footnote c (above) and 4.9 reported in Fujiki and Zurek (1975).

could be due to differences in the strains from which σ was isolated. Fujiki & Zurek (1975) apparently used strain AJ7, rif-rJ7, a heavily mutagenized multiply marked K12 strain, whereas the σ used in this study was from *E. coli* K12 (ATCC 14948) cells (Lederberg strain W3100) containing very few markers.

E. Isoelectric Focusing. Two-dimensional electrophoresis of purified RNA polymerase when carried out exactly as described by O'Farrell (1975) gave unsatisfactory results for σ and β' subunits, Figure 7i (Friesen et al., 1976; Hayward & Fyfe, 1978). σ appeared as a long smear and β' remained at the top of the gel. Modification of the anode buffer allowed β' to enter the gel and produced a sharp major σ band trailing a minor protein (Figure 7ii,iii,v). The modified anode buffer resulted in a slightly lower pH and steeper pH gradient at the acidic end of the gel (Figure 7). β', β, α, and σ migrated to pH 6.8 (top), 5.6, 5.35, and 5.1, respectively, in the unmodified O'Farrell system and to pH 6.9, 5.4, 5.1, and 4.8 in the modified system. A minor protein band is observed which is slightly less negatively charged than the major σ band but has an identical mobility to σ on the Tris–glycine sodium dodecyl sulfate dimension (Figure 7v). It is not present in purified core enzyme preparations (Figure 7iv). This band may represent an impurity, an isoelectric focusing gel artifact (O'Farrell, 1975), a modified form of σ present in vivo, or a form generated artifactually during purification.

This increased clarity of σ isoelectric focusing has facilitated screening of putative σ mutants for charge differences in their polypeptide chain (Burgess et al., unpublished results).

F. Recovery of Activity by σ after Denaturation in 6 M Guanidine Hydrochloride. No noncovalent structure exists in polypeptide chains in 6 M guanidine hydrochloride (Tanford et al., 1967). Many enzymes, including core polymerase

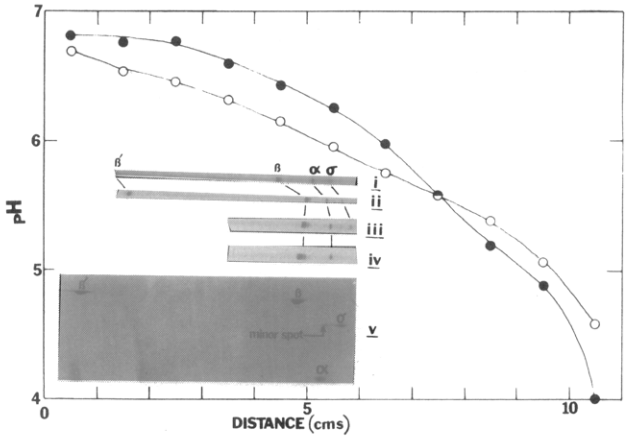

FIGURE 7: Isoelectric focusing properties of σ. pH gradients of standard O'Farrell first-dimension isoelectric focusing gels (○) and gels using the modified anode buffer (●). Points represent the average of three determinations. The inset shows first-dimension (isoelectric focusing) tube gels of (i) holoenzyme using the O'Farrell (1975) procedure; (ii) holoenzyme with the modified anode buffer; (iii and iv) holoenzyme and core polymerase, respectively, using the modified anode buffer (β' not shown); 3 μg of protein was used throughout; (v) two-dimension analysis of holoenzyme. First dimension is identical with sample ii, above. Second-dimension, electrophoresis on a Tris–glycine sodium dodecyl sulfate–8.75% polyacrylamide slab gel.

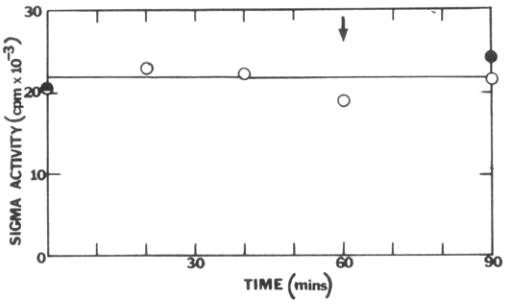

FIGURE 8: Renaturation of denatured σ. σ was denatured by incubation in 0.1 mL of 0.05 M Tris (pH 7.9), 0.1 mM EDTA, 1 mM dithiothreitol, 10 mM $MgCl_2$, 0.1 M NaCl, 20% (v/v) glycerol, and 6 M guanidine hydrochloride for 10 min at 20 °C. A sample was renatured by 50-fold dilution at 0 °C in the above buffer minus guanidine hydrochloride plus 0.5 mg/mL of bovine serum albumin and asssayed immediately for σ activity (zero time point). Incubation of the diluted σ was continued at 0 °C and samples were removed for assay (10 min at 37 °C in the standard assay mix) at the times indicated. After 60 min at 0 °C, incubation was continued for 30 min at 30 °C (shift indicated by arrow). Samples of σ, 0.0075, 0.015, and 0.022 μg, were assayed at various times during the incubation. (○) Activity of 0.0075 μg of denatured and renatured σ. (●) Activity of 0.0075 μg of σ treated in an identical manner, with buffer minus denaturant used throughout. Both 0.0075 and 0.015 μg of σ gave activities on the linear portion of the stimulation curve. Core polymerase activity plus background counts (a total of 745 cpm) was subtracted from total cpm for each point.

(Harding & Beychok, 1974), re-form their catalytic structures after treatment with 6 M guanidine hydrochloride and its subsequent removal. σ denatured in 6 M guanidine hydrochloride and diluted into a buffer without denaturant at 0 °C regained full levels of activity when assayed for 10 min at 37 °C (Figure 8). Further incubation at 0 or 30 °C did not significantly alter the recovery of activity. We do not know if σ renatures fully prior to, or during, the 10-min assay period. However, σ treated with 6 M guanidine hydrochloride, diluted as above, and assayed immediately at 25 °C for 10 min also regained full activity (data not shown). σ interacts with core polymerase and T4 DNA during the assay; hence, these components may bring about a stabilizing effect on σ. Unlike denatured core polymerase, σ does not appear to require

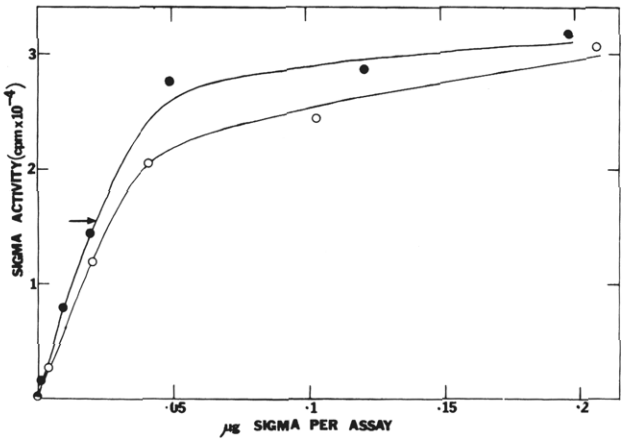

FIGURE 9: Recovery of σ activity from lyophilized powdered σ. σ was precipitated by overnight dialysis at room temperature against double-distilled water (pH 4.8–5.0, adjusted with dilute HCl), lyophilized, and stored as a powder at −20 °C. Powdered σ was dissolved in 0.05 M Tris (pH 7.9), 1 mM EDTA, 2 mM dithiothreitol, 0.25M NaCl, 10% (v/v) glycerol to give a 0.41 mg/mL solution which was diluted prior to assay in the above buffer containing 0.5 mg/mL of bovine serum albumin. (○) Samples of lyophilized and redissolved σ assayed for σ activity in duplicate. (●) σ activity of samples of untreated σ direct from storage. Core polymerase activity plus background counts (a total of 912 cpm) was subtracted from total cpm for each point. Arrow indicates half-maximum stimulation given by the untreated preparation. Protein was determined by the method of Schaffner & Weismann (1973).

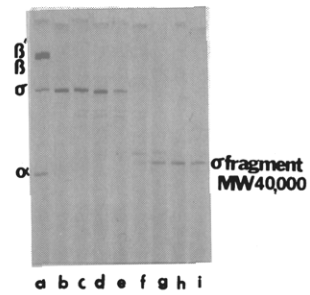

FIGURE 10: Limited proteolysis of σ. Two and a half micrograms of σ in 0.1 mL of 0.01 M Tris-HCl (pH 7.9), 0.1 mM EDTA, 1 mM dithiothreitol, 0.05 M NaCl, 10% (v/v) glycerol was incubated for 30 min at 25 °C with the following amounts of trypsin: (b) 0, (c) 0.0025 μg, (d) 0.006 μg, (e) 0.0125 μg, (f) 0.025 μg, (g) 0.06 μg, (h) 0.125 μg, (i) 0.25 μg. Proteolysis was stopped by the addition of 5 μL of 6 mg/mL of phenylmethanesulfonyl fluoride in ethanol, followed by 0.1 mL of 0.0623 M Tris-HCl (pH 6.8), 3% sodium dodecyl sulfate, 10% glycerol, 5% 2-mercaptoethanol, 0.001% bromophenol blue and boiling for 3 min. Samples were loaded directly onto Tris–glycine sodium dodecyl sulfate–10% polyacrylamide gels and subjected to electrophoresis with an untreated holoenzyme marker, gel (a).

lengthy incubation (approximately 1 h at 37 °C, Harding & Beychok, 1974) in order to achieve a renatured active form. Partially active σ may be recovered from sodium dodecyl sulfate–polyacrylamide gels after elution and subsequent removal of denaturant (Weber & Kuter, 1971; Gross et al., 1978).

Lyophilized, stored σ powder regained high levels of activity when redissolved and assayed (approximately 80% of the specific activity of control σ, Figure 9). In view of the good recovery of σ upon column chromatography during its purification, the rapid return of activity after denaturation–renaturation, and the ability to be lyophilized and redissolved without significant activity loss, it appears that σ is a relatively stable protein. An early report that σ was quite unstable during preparation, manipulation, and storage (Burgess & Travers, 1971) was most likely due to σ sticking to glass tube walls.

G. Proteolytically Generated Fragments. The production of cleaved polypeptides by limited proteolysis of multifunctional proteins has provided valuable insights into the apportioning of function to particular parts of the polypeptide chain (Geisler & Weber, 1977). Limited proteolytic digestion of σ with increasing amounts of trypsin under nondenaturing conditions generated a well-defined series of major fragments (Figure 10). With high levels of trypsin a relatively protease-resistant fragment with a molecular weight of approximately 40 000 was formed. The functional properties of these fragments are under investigation. Storage of σ for months has occasionally resulted in the formation of small amounts of similar fragments. We, therefore, recommend storage of σ at −20 or −70 °C in 0.5 M NaCl and 50% glycerol. Under these conditions of salt and glycerol, σ remains uncleaved after incubation for 90 min at 37 °C with 2×10^{-2} parts (by weight) trypsin per part σ (data not shown).

Added in Proof

We have recently modified the σ purification procedure reported here to remove the 110 000-dalton contaminant more effectively. This increases the yield of the highest purity σ. The DEAE column containing bound σ is washed with TGED + 0.1 M NaCl as before and σ (essentially free of the 110 000-dalton polypeptide) is eluted with TGED + 0.25 M NaCl. The 110 000-dalton polypeptide begins to elute at TGED + 0.26 M NaCl.

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