Chemical Structure of a Modification of the *Escherichia coli* Ribonucleic Acid Polymerase α Polypeptides Induced by Bacteriophage T₄ Infection*

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**SUMMARY**

The α-polypeptides of *Escherichia coli* RNA polymerase are known to be chemically modified within 4 min after infection by bacteriophage T₄. This paper reports a crude system derived from T₄-infected *E. coli* which will modify the α-polypeptides of purified *E. coli* RNA polymerase *in vitro*. The product of this *in vitro* reaction is identical with the modified α found *in vivo*; it differs chemically from the "altered" α observed after T₄ infection in the presence of chloramphenicol. The *in vitro* reaction is rapid, being 50% complete within 30 s at 37°C; the enzyme activity responsible appears less than 2 min after infection at 30°C. These data on kinetics of synthesis and activity are consistent with data on kinetics of α modification *in vivo*.

Specifically labeled radioactive substrates have been used in this *in vitro* modification reaction to investigate the chemical substitution introduced during α modification. The chemical stability of the modification and the sequence of primer peptides containing the modified region of α also have been examined to complement the information obtained with the *in vitro* system. Modification involves covalent attachment of 1 adenine nucleotide, apparently adenosine diphosphoribose, to a specific arginine in the α-polypeptide at the sequence Thr-Val-Arg. NAD+ serves as the donor of 1 adenine nucleotide, apparently adenosine diphosphoribose, to a specific arginine in the α-polypeptide at the sequence Thr-Val-Arg. NAD+ serves as the donor of this nucleotide in the *in vitro* reaction. The α modification is hypothesized to involve adenosine diphosphoribose linked through its terminal ribose to a guanido nitrogen of arginine.

RNA synthesis in bacteriophage T₄-infected, as well as in uninfected, *Escherichia coli* cells is carried out by DNA-dependent RNA polymerase (ribonucleoside triphosphate : RNA nucleotidyltransferase, EC 2.7.7.6). A key role for this enzyme in *T₄*-infected cells was first shown by the observation that the antibiotic rifampicin, which stops all *E. coli* RNA synthesis by binding to RNA polymerase (1, 2), also stops all RNA synthesis in *T₄*-infected cells (3). This was a surprising observation initially since many important changes in transcription specificity take place after *T₄* infection. Host RNA synthesis rapidly stops and a well defined series of changes in the pattern of *T₄* DNA transcription ("immediate early" to "delayed early" to "late" RNA) occurs (4-11). Although the molecular basis for most of these changes is still uncertain, it will be very surprising if some of the transitions do not result from changes that have recently been observed in several of the polypeptides which make up the RNA polymerase ββ′αα₁α₂α₃ subunit structure (12, 13).

The first of these changes in RNA polymerase to be discovered is in the α subunits. Within 4 min after *T₄* infection the two 40,000-dalton α-polypeptides become more negatively charged, as shown by their altered electrophoretic mobility in both alkaline and acid polyacrylamide gels containing urea (14-17). In this process the pre-existing α-polypeptides are modified rather than replaced (17). Modified α contains covalently linked phosphorus, and 5′AMP can be released from the modified structure with venom phosphodiesterase (17). The covalent phosphorus is associated with a unique tryptic peptide from modified α (18), suggesting that a specific sequence in the polypeptide is affected.

This paper further investigates the enzymology and structure of the *T₄*-induced α modification.

**EXPERIMENTAL PROCEDURES**

**Materials**

SDS,1 phenylisothiocyanate, and dansyl chloride (all sequential grade) were from Pierce Chemical Co., Rockford, Ill. Urea (ultrapure) was purchased from R-Plus Labs, Donville, N. J. Acrylamide, N,N-methylene bisacrylamide, and N,N,N′,N′-tetramethylethylenediamine (all Electrophoresis grade) came from Bio-Rad Labs, Richmond, Calif. All other chemicals were reagent grade.

*NAD* labeled with ³²P in both phosphates, 100 to 200 Ci per mole, was prepared by the method of Colowick and Kaplan (19) as modified by GHL (20). Approximately 45% of total ³²P in this *NAD* was in the nicotinamide-proximal phosphate, and 55% in the adenine-proximal phosphate, as judged by chromatography

1 The abbreviations used are: SDS, sodium dodecyl sulfate; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; TES, N-tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid.
after cleavage with venom phosphodiesterase. NAD$\textsuperscript{+}$ labeled with $^{14}$C in the ribose part of NMM, approximately 100 Ci per mole, was synthesized enzymatically from [U-$^{13}$C]glucose (American, $290$ Ci per mole) essentially by the method of Ueda and Yamamura (21). Cleavage with venom phosphodiesterase proved that 100% of the label was in the NMM moiety. 

adenosine-G-H-NAD$^+$, $1000$ Ci per mole, and [nicotinamide-4-H-NAD$^+$, $250$ Ci per mole, were purchased from New England Nuclear; [nicotinamide-7-$^{13}$C-NAD$^+$, $50$ Ci per mole, came from Amersham.

Rabbit antisera against purified Escherichia coli RNA polymerase was prepared by conventional immunization techniques; typically 1 ml of serum quantitatively precipitated 100 $\mu$g of RNA polymerase. Immunoglobulins were partially purified from the crude serum by passage over DEAE-Cellulose (Whatman DE52) in $25$ mM sodium phosphate, pH 7.2, followed by precipitation with ammonium sulfate (added to 50% of saturation). The precipitate was dissolved at $10$ mg per ml of protein and stored at 4°C in 50 mM sodium phosphate, pH 7.2, 75 mM NaCl, and 0.1 mM EDTA.

E. coli B/r T1, Su$^+$ and prototrophic, was obtained from Dr. R. Schleif, Brandeis University (his strain RFS 01). T1D came from Dr. M. Meselson, Harvard University. TdD6 (amber mutant in gene e, lysozyme) and its permissive host E. coli B/r (Su I) were purchased from TaH26 (amber mutant in gene a, polar) from Dr. M. Meselson, Harvard University. 

**In Vitro** $\alpha$-Modifying System

Growth of $T_1$-Infected Cells

E. coli B/r is grown at 30°C with vigorous aeration in a medium containing, per liter: 5 g of NaCl, $12$ g of Difco yeast extract, and 20 g of Difco Bacto-tryptone, adjusted to pH 7.2 with NaOH. When the cells reach a density of 8 to 10$^9$ per ml they are infected with $T_1$ phage at a multiplicity of 5 to 8, grown 3 to 4 min longer, and poured over 0.5 volume of frozen M8 buffer (22). The infected cells are quickly centrifuged out, washed once by resuspending in 250 ml of cold wash buffer (40 mM TES, pH 7.4-10 mM MgCl$\textsubscript{2}$-50 mM KCl, 20% glycerol (v/v), 0.2 mM EDTA, and 0.2 mM dithiothreitol), and pooled. The lysates are brought to 15 mM MgCl$\textsubscript{2}$ and washed, the precipitate is dissolved in 100 ml of cold 50 mM Tris-Cl, pH 7.5. After two more cycles of pelleting and washing, the precipitate is dissolved in 100 ml of cold 50 mM Tris-Cl, pH 7.2, 5% β-mercaptoethanol, 10% glycerol, and 3% SDS), heated 2 min at 30°C to denature antigen-antibody complexes, and subjected to electrophoresis on a 6.5% acrylamide gel containing 0.1% SDS (24).

Following electrophoresis the gel is sliced and prepared for scintillation counting as previously described (17). The peak of counts undergoing electrophoresis is in the position of $\alpha$ is summed to determine incorporation in the reaction.

Large Scale Preparation of Core RNA Polymerase and Its $\alpha$-Polypeptide from T1-infected Cells

**Growth of Cells**—Large cultures of E. coli B/r are grown in a 250-liter fermentor, in a medium containing, per liter: 5 g of NaCl, 6 g of Difco yeast extract, 5 g of Difco Bacto-tryptone, and 10 g of casein hydrolysate (Pepitone No. 50, General Biologicals, Chagrin Falls, Ohio). The cultures are grown at 30°C with vigorous aeration to a cell density of 1.2 X 10$^9$ per ml, then infected with $T_1$H96 phage at a multiplicity of 6 to 8. The infected cells are grown 10 to 15 min, chilled, and harvested in a refrigerated continuous flow centrifuge. Use of phage unable to synthesize lysozyme in these nonpermissive cells reduces the fragility of the infected cells and prevents losses due to lysis during harvest. The harvested infected cells are grown identically but without addition of phage.

**Purification of RNA Polymerase**—“Core” RNA polymerase is purified from T1-infected and normal cells by the phosphocellulose procedure of Burgess (25), modified as follows. A 30 to 50% saturated ammonium sulfate fractionation of the high speed supernatant (Burgess's Fraction 9) is used, followed by back extraction with 42% saturated ammonium sulfate in Burgess's Buffer C. Column chromatography on both DEAE-cellulose and phosphocellulose utilizes Buffer C; columns are eluted with steep gradients of KCl rather than steps. For DEAE-cellulose, the elution gradient (total of 5 column volumes) runs from 100 to 500 mM KCl; for phosphocellulose it runs from 150 to 400 mM KCl, again in 5 column volumes.

To remove residual nucleic acids and thus assure binding of the enzyme to phosphocellulose, a phase-partitioning step (26) has been added. Active fractions from the DEAE-cellulose column are pooled and concentrated by precipitation with ammonium sulfate (added to 90% of saturation). The precipitate is re-suspended and dialyzed into Buffer C (final volume, 75 ml for a preparation from 500 g of cells). This solution is made 4 n in NaCl, 6.9% (w/v) in polylethylene glycol 6000, and 1.75% (w/v) in dextran T500 (Lot 5207, Pharmacia); the resulting two-phase system is stirred 1 hour at 4°C. The phases are separated by centrifugation (100 X g, 20 min) and the top phase is withdrawn. The viscous bottom phase is re-brought up to 0.5 ml of Buffer C made 4 n in NaCl and 6.9% in polylethylene glycol and the phases again are separated. The first and second upper phases are combined and dialyzed against Buffer C until the conductivity equals that of Buffer C plus 120 mM KCl. The gummy precipitate formed during dialysis is centrifuged out and discarded; the clear supernatant is loaded onto a phosphocellulose column equilibrated with Phosphate Buffer (100 mM KCl, Polylethylene glycol flows through the column and does not interfere with chromatography.

After phosphocellulose chromatography RNA polymerase preparations from T1-infected cells contain more protein impurities than do preparations from normal cells. Therefore velocity gradient centrifugation rather than Burgess's agarose column is used to resolve the RNA polymerase more completely.
from contaminating proteins in the final sizing step. The active fractions from the phosphocellulose column are pooled and precipitated by dialysis against 75% saturated ammonium sulfate in Buffer C. The precipitate is resuspended and dialyzed into Buffer C plus 250 mM KCl (final protein concentration, about 25 mg per ml). Two milliliters of this solution are layered onto a 30-ml gradient of 10 to 30% (v/v) glycerol in Buffer C plus 250 mM KCl, and centrifuged at 27,000 rpm in a SpincO SW-27 rotor. Twenty-five fractions are collected from each gradient.

The RNA polymerase from these gradients is free of measurable impurities, as judged by electrophoresis in SDS- or urea-containing polyacrylamide gels. Final yield of core enzyme is about 250 mg per kg of wet cells with normal E. coli, and about 150 mg per kg with phage-infected cells.

The α-polypeptide of the enzyme from different batches of infected cells is 80 to 100% modified, as judged by the mobility of α in pH 4.4 polyacrylamide gels containing urea (see below).

**Purification of RNA Polymerase α-Polypeptide**—The α-polypeptide is sufficiently different in molecular weight from β, β′, γ, and the T4-specific polypeptides associated with RNA polymerase after infection (27) that SDS gel electrophoresis can purify it completely. Large preparative SDS gels (0.0 × 15 cm; 8% acrylamide) are cast in glass tubes and thoroughly washed prior to electrophoresis as described by Weiner et al. (28) to remove insoluble material which otherwise interferes with chemical analysis of the purified protein.

RNA polymerase to be used as a source of α is precipitated by dialysis against 75% saturated ammonium sulfate in Buffer C. The precipitate is redisolved in a small volume and dialyzed against gel loading buffer containing no SDS. The solution is adjusted to 10 mg per ml of protein, made 3% in SDS, and heated 1 min at 95°. Up to 5 mg of RNA polymerase can be loaded on each gel. Stacking is carried out at 6 mA per gel for 1 hour, and the current is then increased to 10 mA per gel. Total electrophoresis time is normally 18 hours. The electrophoresis buffer is replaced when the pH of the upper and lower buffer chambers of the gel apparatus differ by more than 1 unit.

Following electrophoresis, the position of α-polypeptides in the gel is easily determined by chilling the gel for 2 hours at 4°. Protein-SDS complexes are insoluble at 4° and precipitate in the gel, forming opaque bands. The broad α-band (mobility about 0.7 relative to bromphenol blue) is sliced out and the gel section (about 1.5 cm long) is crushed with a spatula, then shaken gently at 25° for 8 hours with 2.5 ml per gel of gel eluting buffer (50 mM NH₄HCO₃, 0.1% SDS). The fragments of polyacrylamide are centrifuged out (2500 × g, 5 min) and the supernatant is removed; Total electrophoresis time is normally 18 hours. The electrophoresis buffer is replaced when the pH of the upper and lower buffer chambers of the gel apparatus differ by more than 1 unit.

**Preparation of Radioactively Labeled RNA Polymerase α-Polypeptide**

**Growth of Cells**—Small cultures of E. coli B/r for radioactive labeling, usually 25 to 50 ml, are grown in Erlenmeyer flasks in a shaking water bath at 30°. 32P labeling is done in low phosphate nH 7.4. 40 mM KCl and NaCl, 10 mM each: NH₄OH, 20 mM: MeSO₄. labeling, usually 25 to 50 ml, are grown in Erlenmeyer flasks in a shaking water bath at 30°. 32P labeling is done in low phosphate

**Double labeling with [32P]phosphate and [3H]proline is carried out identically, except that proline is omitted from the low phosphate medium, and 1-15-NH₃ proline, 40,000 Ci per mole (Schwarz-Mann); is added in three aliquots, 5 min apart, prior to phase infection. About 2 × 10⁶ dpm are incorporated into α from 250 µCi of [3H]proline.**

**Purification of Radioactively Labeled RNA Polymerase α-Polypeptide**—The experiments reported here require complete radiochemical purification of α-polypeptide containing about 50 nCi of stable 32P from T4-infected cells containing up to 10 microcuries of organic 32P. This degree of purification necessitates removal of most nucleic acids before even the powerful technique of antibody precipitation can be used.

Frozen radioactively labeled cells (25 to 50 mg) are mixed with approximately 0.4 g of unlabeled cells grown under similar conditions. Phage-infected cells are resuspended in 5 ml of cold lysis buffer containing 0.5 mg per ml of lysozyme for 20 to 30 min, three cycles of freezing and thawing in a Dry Ice-acetone bath and 37° water bath. The viscous lysate is digested with DNase I (3 µg per ml) for 2 min at 37°, then made 2% in glycerol and 200 mM in KCl to prevent aggregation of RNA polymerase. All subsequent steps are performed at 4°. The extract is centrifuged at 37,000 rpm for 1.5 hours in a Spinco angle 40 rotor. One volume of saturated ammonium sulfate solution (25°) adjusted to pH 7.0 with NH₄OH is added to the ribosome-free supernatant. The solution is mixed and allowed to stand 20 min, then centrifuged (7,500 × g, 15 min); the supernatant is discarded and the precipitate, containing the RNA polymerase, is washed in 1.5 ml of Buffer C plus 5 mM KCl, and centrifuged 12 hours at 49,000 rpm in a SpincO SW-50 rotor. Twenty fractions are collected and those containing material sedimenting at 10 to 17 S are pooled.

The protein in this fraction is approximately 25% pure RNA polymerase, but up to 90% of the phosphorus present is still in nucleic acid, rather than covalently associated with RNA polymerase. Final purification of the labeled T4-modified α-polypeptide is achieved by specific precipitation of the RNA polymerase with antibody, denaturation of the antibody-antigen complexes with 6M SDS, and electrophoresis on 6.5% SDS polyacrylamide gels, as described above for analysis of in vitro modification reactions. The antigen-antibody complex from a preparation of 0.5 g of cells (containing about 100 µg of RNA polymerase) can be subjected to electrophoresis on one 6-mm diameter gel, and the position of the α-polypeptides is easily visualized by chilling the gel as described above for preparative purification of unlabeled α-polypeptide. The α-band is sliced out and the gel section (about 5 mm long) is eluted with 1 ml of gel eluting buffer as described above. On a 10-cm long 6.5% polyacrylamide gel, α is completely resolved from immunoglobulin polypeptides; this method yields 32P-labeled α more than 90% pure both radiochemically and chemically.

**Production and Purification of Pronase Peptides from T₄-modified α-Polypeptide**

To assure complete digestion by pronase, and to simplify subsequent purification of the resulting peptides, purified α is freed of SDS and digested in urea. Approximately 4 mg of T₄-modified α-polypeptide (0.1 µmole) and a few thousand disintegrations per minute of 32P-labeled in vivo modified α, both lyophilized with SDS, are dissolved in 750 µl of 6 M urea-75 mM (NH₄)HCO₃ and passed slowly over a 1-ml column of Dowex 1-X8 (acetate form) equilibrated with the same buffer. Fractions containing radioactive material (about 1.5 ml); 20% of input α is recovered free of SDS (29).

Forcytromicrographs of pronase (nuclease-free, B grade, Lot 029352; Calbiochem) are added to the solution, which then is diluted to 3 M urea with 75 mM (NH₄)HCO₃ and incubated at 25°. After 6
hours another 40 µg of pronase are added and digestion is continued for 6 to 8 hours more.

After digestion, the peptide solution is lyophilized, redissolved in water, and relyophilized to remove (NH₄)₂HCO₃. The peptides are redissolved in 2 ml of water and passed slowly over a 1-ml column of Dowex 50-X2 (H⁺ form). ³²P covalently linked to peptides is retained by the column; free phosphate and nucleotides flow through. Residual urea is washed through the column with water, and the peptides then are eluted from the column with ice-cold 1.0 N NH₄OH. Fractions containing radioactivity are pooled and quickly lyophilized. About 80% of the ³²P label originally in α is recovered in peptides.

The lyophilized pronase peptides are dissolved in 100 µl of water and applied to a sheet of Whatman No. 3MM paper (46 × 57 cm) in a band (1 × 3 cm) near one corner. The paper is wetted with pH 1.8 buffer (7% formic acid) and subjected to electrophoresis at 1800 volts for 2 hours in a refrigerated tank. The phosphate-containing peptides are very slow moving at this pH. After drying at room temperature, the sample is concentrated to a narrow band near the edge of the paper sheet by wetting with electrophoresis buffer, dried again, and chromatographed in the second dimension, perpendicular to electrophoresis. Ascending chromatography requires 20 hours at 25° in a closed tank, using (by volume) 7.6% acetic acid, 37.8% 1-butanol, 24.4% pyridine, and 30.2% distilled water. The modified peptides are once again very slow moving (see Fig. 4), presumably due to their high charge density. Following chromatography the paper is dried in a hood.

Phosphate-containing peptides are located by autoradiography on Kodak No-Screen x-ray film; the modified peptides can also be located by the ultraviolet absorption of the adenine which they contain, if at least 10⁻⁴ moles are present. Complete purification requires a third separation; a rectangle (3 × 6 cm) containing the ³²P-labeled peptides is cut from the paper, stitched into a new sheet, and subjected to electrophoresis at pH 3.5 (in 5.0% acetic acid, 0.5% pyridine). After this electrophoresis (2500 volts, 5 hours) the labeled peptides again are located by autoradiography. Radioactive spots are cut from the sheet and soaked in 2 ml of 0.2 N acetic acid for 12 hours at 25° to elute the peptides. The supernatants are lyophilized.

Other Methods

Sequence Determination—Peptides are sequenced by the microdansyl Edman method of Hartley (30) as modified by Platt et al. (31).

Amino Acid Analysis—Amino acid compositions of peptides are determined on a Beckman 110 analyzer with expanded recorder range. Aliquots of peptides (2 to 5 nmole) are hydrolyzed at 105° for 20 to 24 hours in sealed evacuated tubes with 20 µl of 6 N HCl, dried down, and applied to the analyzer columns. Assignment of components and amides is made by dansylation following digestion of peptides with leucine aminopeptidase. Peptides (0.2 to 0.5 nmole) are dried down in tubes (6 × 30 mm), taken up in 5 to 10 µl of 50 mM NaHCO₃, 10 mM MgCl₂ containing 1 mg per ml of leucine aminopeptidase (Worthington, code LA1PC), and digested. 12 hours at 37°. One-half volume of 0.5% dansyl chloride in acetone is added to the digest; after incubation for 30 min at 37°, the dansyl-amino acids are identified as usual on polyamide sheets (28). Leucine aminopeptidase does not interfere with the chromatography and does not generate significant background by self-digestion.

Analytical Polyacrylamide Gel Electrophoresis—SDS gel electrophoresis (24) for estimation of protein purity is carried out on 6.5% polyacrylamide gels. Estimates of the degree of α modification are made using pH 4.4 polyacrylamide gels containing 5% urea-3% d-mercaptoethanol-0.0001% basic fuchsin (tracking dye); and the electrophoresis buffer, adjusted to pH 4.4, is diluted 4-fold. Electrophoresis is at room temperature and 2.5 ma per gel. All gels are stained with Coomassie brilliant blue R-250 as described by Burgess (25), then destained by gentle shaking with a small quantity of AG-50I mixed bed resin (Bio-Rad Laboratories) in 7.5% acetic acid-5% methanol for 12 hours at 37°. Semi-quantitative estimates of the degree of α modification are made by scanning stained pH 4.4 gels at 600 nm in a Gilford scanning spectrophotometer and comparing the area under modified and unmodified α-peaks.

Results

Design of In Vitro α Modification Assay—The in vitro modification assay described under “Experimental Procedures” was designed on the basis of the known timing of α modification in vivo (16) and the proven presence of an adenine nucleotide in the modified polypeptide (17). Thus 3- to 4-hour-infected cells were chosen as a source of the modifying activity, and NAD⁰ and ATP were both tested as potential nucleotide donors for the reaction. In the absence of data on the properties of an α-modifying activity, essentially unfractonated cell lysates were used. Antibody precipitation and polyacrylamide gel electrophoresis were chosen as rapid, simple methods of removing unincorporated label and assuring the specificity of the assay. It was obvious that a very sensitive assay such as this would be needed since both ATP and NAD⁰ are present at over 1 mM concentration in Escherichia coli (33, 34) and the specific activity of the added radioactive material would be greatly diluted in a crude extract.

Incubation of E. coli RNA polymerase with a concentrated extract of infected cells and adenine-labeled NAD⁰ as described results in incorporation of about 0.05% of the input label into material undergoing electrophoresis in the position of modified α on the SDS polyacrylamide gel (Fig. 1). There is no incorporation of ATP label into α under these conditions (data not shown).

Characterization of System—This assay measures incorporation of label into the α-polypeptide and not a contaminant. The labeled product sediments with RNA polymerase in velocity gradients (data not shown), is specifically precipitated by anti-RNA polymerase antibody, and undergoes electrophoresis with modified α in SDS gels. Furthermore incorporation is largely dependent on added pure RNA polymerase (see discussion of Fig. 3, below).

![Image](https://via.placeholder.com/150)

FIG. 1. The product of the in vitro modification reaction undergoes co-electrophoresis with in vivo-modified α. A standard in vitro modification reaction was carried out and analyzed as described under “Experimental Procedures” (extract prepared by Method A) except that a small amount of [³⁵S]-labeled RNA polymerase (gift of Dr. A. E. Sippel, Harvard University) was added to the gel loading buffer before heating, and the gel was coated for both [³⁵S] and °H. Parallel stained gels predicted normal α to be in Slices 39 and 40, and in vitro-modified α to be in Slices 38, 39, and 40. This slight mobility difference (17) is reflected in the distribution of [³⁵S] (normal) and °H (in vitro-modified) α. O, [³⁵S]; °H.
Isotope dilution of the input label with nonradioactive NAD\(^+\) indicates that as expected the in vitro reaction contains approximately 0.4 mM endogenous NAD\(^+\) (data not shown). Attempts to reduce endogenous NAD\(^+\) and thereby increase the specific activity of label, either by dilution of the crude extract or by adsorption of NAD\(^+\) on Norit, have thus far caused loss of the modifying activity.

Despite the low level of incorporation and the extensive workup involved, the assay is reproducible to better than \(\pm 10\%\) with any one extract. Different extracts from 3- to 4-min infected cells may vary up to 3-fold in activity; the most active extracts stimulate incorporation of about 0.5 mole of adenosine per mole of input \(\alpha\)-polypeptide. The presence of initiation subunit \(\alpha\) in the added RNA polymerase has no effect on incorporation of label into \(\alpha\) (data not shown).

The in vitro \(\alpha\)-modifying activity is unstable in a crude extract, with a half-life of about 8 min at 37\(^\circ\) C (data not shown). The in vitro reaction is rapid, being 50% complete within 30 s at 37\(^\circ\) C, and the product is stable indefinitely in the crude extract, long after the modifying activity has disappeared (Fig. 2). The reaction is apparently irreversible under these conditions, since chasing the label with 2.5 mM NAD\(^+\) at 5 min (after the reaction is complete but before the activity has disappeared) causes no decrease in final incorporation after 15 min (373 cpm incorporated in normal reaction; 382 cpm with chase at 5 min).

The activity being assayed in this in vitro reaction is clearly phage-induced. Activity is found only in infected cells and appears rapidly after infection with kinetics expected from data on \(\alpha\) modification in vivo. This was shown by assaying extracts made from cells harvested at various times after infection (Fig. 3, filled circles). If these same extracts are assayed without added RNA polymerase, some incorporation is observed very early after infection (Fig. 3, open circles). I assume that this incorporation results from labeling of RNA polymerase already present in the extract (estimated to be about 20 \(\mu\)g per reaction).

The \(\alpha\)-polypeptides, once modified in vivo, apparently cannot accept label in vitro; if modified RNA polymerase purified from T\(_4\)-infected cells is added to an in vitro modification reaction instead of the normal enzyme, essentially no labeling of its \(\alpha\)-polypeptides occurs (data not shown). This presumably explains why the endogenous RNA polymerase can accept label at 2 min after infection, but not by 4 min; \(\alpha\) modification is known to be underway in vivo at 2 min and complete by 4 min (16). In vitro activity towards added RNA polymerase alone (the difference curve of Fig. 3, squares) is only slightly diminished between 2 and 4 min, but disappears by 12 min. Thus the enzyme(s) involved evidently remain active for a short time after RNA polymerase has been completely modified in vivo.

In Vitro and In Vivo Modifications Are Identical—Since in vivo modification apparently prevents in vitro labeling, as discussed above, the in vivo and in vitro reactions could involve the same site in \(\alpha\). If we can show that the product of the in vivo reaction is chemically identical to the modification produced in vivo, the in vivo system will provide a convenient tool for studying the structure of the \(\alpha\) modification. I have compared the radioactive peptides produced by pronase digestion of in vivo-modified and in vitro-labeled \(\alpha\)-polypeptides to prove their identity.

For this experiment \(^{32}\)P-labeled in vivo-modified \(\alpha\) was prepared from infected cells grown in medium containing \(^{32}\)P-phosphate, as described under "Experimental Procedures." \(^{32}\)P-labeled in vitro modified \(\alpha\) was prepared in a standard in vitro modification reaction with \(^{32}\)PNAD\(^+\). The in vivo- and in vitro-labeled \(\alpha\)-polypeptides were purified in parallel on SDS gels and recovered. Pronase peptides of the in vivo- and in vitro-labeled polypeptides were prepared and fingerprinted in parallel by \(\mathrm{pH}\) 1.8 electrophoresis and chromatography as described under "Experimental Procedures." Autoradiography revealed that over 90% of \(^{32}\)P moved as a single spot in the same position on both fingerprints (Fig. 4, A and B). In both cases that spot was resolved into two spots by \(\mathrm{pH}\) 3.5 electrophoresis, and again the mobilities of in vivo- and in vitro-labeled peptides were identical (Table I). The same result was also obtained by digesting a mixture of \(^{32}\)P-labeled in vivo-modified \(\alpha\) and \([\text{adenosine-G-\(\beta\)}]^{32}\)P-NAD\(^+\) in vitro-labeled \(\alpha\); a single radioactive spot containing both \(^{32}\)P and \(^{3}\)H labels was found on the two-dimensional fingerprint. Since the radioactive peptides from in vivo- and in vitro-modified \(\alpha\) behave identically in chromatography and electrophoresis, the substitutions must be the same.

It should be noted here that the product of the in vivo and in vitro modification reactions differs from the partially "altered" \(\alpha\)-polypeptides found in vivo after T\(_4\) infection when phase protein synthesis is prevented by chloramphenicol (18). This "alteration," like modification, involves covalent addition of phosphorus and adenosine to \(\alpha\), but different \(^{32}\)P-labeled peptides were observed in tryptic digests of in vivo-labeled "altered" and modified \(\alpha\) (18). Reproducibly different patterns of radioactive pronase peptides are also obtained from "altered" and modified \(\alpha\) (Fig. 4C). The chemical and enzymatic relationship between
"alteration" and modification of α is still unclear, but the in vitro reaction is unambiguously modification. Therefore specifically labeled NAD+ can be used in the in vitro system to determine what portion of the NAD+ molecule is transferred to α during modification.

Both Phosphates of NAD+ Are Covalently Attached to Modified α—To distinguish whether one or both phosphates of NAD+ are donated to α during modification, the following experiment was performed. NAD+ doubly labeled with 3H in adenosine and 32P in both phosphates was used to modify α in a standard in vitro reaction. The reaction was analyzed as usual and the SDS gel was counted for both isotopes. An aliquot of the same doubly labeled NAD+ was incubated with diphtheria toxin and rabbit peptidyl-tRNA translocation factor EF-2. Diphtheria toxin catalyses covalent transfer of adenosine diphosphoribose from NAD+ to the EF-2 polypeptide (35) so this system provides a standard incorporating adenosine and both phosphates. This reaction was analyzed on an SDS gel in parallel with the modified α. Because input label and counting conditions were identical, the ratio of counts of 32P to counts of 3H in α and in EF-2 can be compared directly to determine the number of phosphorus atoms per adenosine present in modified α. The 32P/3H ratios in α and EF-2 are essentially identical (Table II), so both phosphates from NAD+ must be incorporated into modified α. This result is consistent with a previous report on the phosphate content of in vivo-modified α (18).

Adenosine Diphosphoribose, but Not Nicotinamide, Appears To Be Present in Modified α—Two additional double-label experiments have been carried out to further define the nucleotide substituent on modified α. Two in vitro α modification reactions were prepared. Reaction A contained a mixture of [adenosine-3H]NAD+ and [14C]NAD+ labeled in the ribose carbons of the NMN half; Reaction B contained [adenosine-3H]NAD+ and [nicotinamide-7-14C]NAD+. These reactions were analyzed as usual and counted for both isotopes. As is clear from Table III, Reaction A incorporated equal proportions of adenosine and NMN-ribose label, while Reaction B incorporated adenosine but essentially no nicotinamide label. This negative result for nicotinamide incorporation was confirmed by testing incorporation of a mixture of [nicotinamide-4-3H]NAD+ and [14C]NAD+; 32P was incorporated (240 cpm per 106 cpm input), but again nicotinamide label was not (7 cpm per 107 cpm input). Thus, label from all parts of the NAD+ molecule except nicotinamide is found in α after modification.

The nearly equal efficiency of 14C and 3H incorporation in Reaction A of Table III suggests that all of the NMN-ribose carbon atoms (presumably uniformly labeled in this [14C]NAD+) are attached to α. The simplest interpretation of these results is that

![Image](http://www.jbc.org/)

**Fig. 4.** Pronase peptide fingerprints of in vivo- and in vitro-labeled modified α and labeled "altered" α. a-32P-Labeled in vivo-modified α-polypeptide was prepared as described under "Experimental Procedures." a-32P-Labeled "altered" α was prepared identically from 32P-labeled cells infected in the presence of 400 μg per ml chloramphenicol (added 5 min prior to infection) and washed in medium also containing chloramphenicol. a-32P-Labeled "altered" α was prepared in a standard in vitro modification reaction (see Fig. 4). Reaction was analyzed on an SDS gel in parallel with the in vivo-labeled α. Approximately 400 μg of normal Escherichia coli a-polypeptide was added to each of the labeled preparations as carrier, and the preparations were freed of urea and fingerprinted by pH 1.8 electrophoresis and chromatography, again as described under "Experimental Procedures." Following digestion the peptides were freed of urea and fingerprinted by pH 1.8 electrophoresis and chromatography, again as described under "Experimental Procedures." The fingerprint maps were dried and exposed to Kodak No-Screen x-ray film. A, autoradiogram of labeled pronase peptides from "altered" α. Dimension 1, pH 1.8 electrophoresis; Dimension 2, ascending chromatography. The spot contains peptides Pro 1 and Pro 2 (see Table I). B, autoradiogram of labeled pronase peptides from in vivo-modified α. Spot contains peptides In vivo 1, In vivo 2 (Table I). C, autoradiogram of labeled pronase peptides from "altered" α. D, pattern of unlabeled peptides from α in the fingerprints. The fingerprint map autoradiographed in A was sprayed with 0.5% ninhydrin and photographed to reveal the positions of unmodified peptides. (Only acidic and neutral peptides are visible here; basic peptides have moved off the paper.)

| Peptide | Electrophoretic mobility at pH 1.8 | Chromatographic mobility (solvent front = 1.0) | Electrophoretic mobility at pH 14 | (basic fuchsin = 1.0) |
|---------|----------------------------------|-----------------------------------------------|---------------------------------|-------------------|
| Pro 1   | 0.50                             | 0.14                                          | 0.26                            |
| Pro 2   | 0.50                             | 0.14                                          | 0.21                            |
| In vitro 1 | 0.51                      | 0.14                                          | 0.25                            |
| In vitro 2 | 0.51                      | 0.14                                          | 0.21                            |
| LAP 1   | 0.39                             | 0.13                                          | 0.18                            |

**TABLE I**

Mobilities of radioactive peptides from 32P-labeled in vivo- and in vitro-modified α-polypeptides

**TABLE II**

| Peptide | Electrophoretic mobility at pH 1.8 | Chromatographic mobility (solvent front = 1.0) | Electrophoretic mobility at pH 14 | (basic fuchsin = 1.0) |
|---------|----------------------------------|-----------------------------------------------|---------------------------------|-------------------|
| Pro 1   | 0.50                             | 0.14                                          | 0.26                            |
| Pro 2   | 0.50                             | 0.14                                          | 0.21                            |
| In vitro 1 | 0.51                      | 0.14                                          | 0.25                            |
| In vitro 2 | 0.51                      | 0.14                                          | 0.21                            |
| LAP 1   | 0.39                             | 0.13                                          | 0.18                            |
Table II

In vitro incorporation of NAD* labeled with *H in adenosine and "P in both phosphates into modified & and diphtheria toxin-inactivated EF-2

NAD* doubly labeled with *H in adenosine and "P in both phosphates was used to modify & in a standard in vitro reaction. An aliquot of the same double-labeled NAD* (about 5 nCi) was incubated 30 min at 37° with 10 µg of partially purified rabbit EF-2 and 1 µg of activated diphtheria toxin (both kind gifts of Dr. D. M. Gill, Harvard University) in 50 µl of 20 mM Tris-Cl, pH 8.0-5 mM dithiothreitol. Unincorporated label was removed by the addition of 5 µg of acid-washed Norit in 50 µl of 10 mM sodium phosphate, pH 7.2. After 15 min at 25° the Norit was centrifuged out. The supernatant, was made 2% in SDS, 3% in glycerol, and 10% in glycerol, heated 2 min at 95°, and subjected to electrophoresis on a 6.5% SDS polyacrylamide gel in parallel with the in vitro-modified &. Both gels were sliced and counted for *P and *H. The peak of counts in the position of EF-2 and & on the respective gels were summed and are presented here for two experiments (in which different batches of ["P, *H] NAD* were used, with different ratios of input "P and *H).

| Experiment | Reaction | Input | Incorporated | Incorporation per 10^-6 input cpm |
|------------|----------|-------|--------------|----------------------------------|
| 1          | A ["C]NAD*, label in ribose of NMN moiety [adenosine-"H]NAD* | 1.5 x 10^4 | 481 | 320 |
| 2          | A [nicotinamide-7,"C]NAD* [adenosine-"H]NAD* | 0.85 x 10^6 | 6 | 7 |

Table III

Adenosine diphosphoribose, but not nicotinamide, appears to be present in in vitro-modified 

Two in vitro modification reactions were prepared (extracts made by Method B) containing NAD* labeled as indicated. They were analyzed as usual and counted for both *H and "C. Efficiency of incorporation of adenosine label differed in Reactions A and B because different extracts were used.

Table IV

Effect of diphtheria toxin on the incorporation of adenosine diphosphoribose into modified &

The approximate concentration of adenosine present in the solution of modified & can be calculated from the difference spectrum and the known ε₉₀ of adenosine. To calculate the concentration of & from its spectrum in Fig. 5A, and thus determine the molar ratio of adenosine to &, we first must obtain a molar extinction value for the unmodified polypeptide. The amino acid composition of normal & was determined by hydrolysis with p-toluenesulfonic acid, which allows direct quantitation of tryptophan on the amino acid analyzer (36). These data indicate 1 tryptophan and 6 tyrosines per 40,000 daltons¹ and thus an ε₉₀ of about 12 x 10⁴ for unmodified &. Finally, we can calculate

¹ Burgess, using spectral methods, has determined 1 tryptophan and 5 tyrosines per 40,000 daltons from the α-polypeptide of Escherichia coli K12 RNA polymerase (37). The discrepancy may reflect either strain differences or errors in one determination.
Modification Is Linked to Arginine in α-Polypeptide—As already mentioned, pronase digestion of 32P-labeled modified α produces two labeled peptides separable by pH 3.5 electrophoresis, Pro 1 and Pro 2 (Table 1). These modified peptides were purified from several milligrams of modified α (see "Experimental Procedures") in order to determine the amino acid involved in the linkage. Pro 1 was recovered from paper in 16% molar yield, and Pro 2 in 7.5% yield, relative to the amount of α originally digested. The amino acid composition of Pro 1 was determined by acid hydrolysis to be: Thr, 0.9; Gly, 0.7; Val, 1.0; and Arg, 1.0. Pro 2 was found to contain: Gly, 0.6, Val, 1.0, and Arg, 1.0. No other amino acids were present at over 0.1 residue. Dansyl-Edman sequencing gave the following results: Pro 1, Thr-Val-Arg; Pro 2, Val-Arg.

Glycine is never obtained as an NH2-terminal amino acid during Edman degradation of either Pro 1 or Pro 2. The presence of glycine in low molar ratio in acid hydrolysates of these modified peptides is an artifact produced during hydrolysis by degradation of the adenine in the modification. Two lines of evidence support this assertion. First, acid hydrolysis of authentic adenine under the conditions used for composition analysis produces glycine in 40 to 60% molar yield. Secondly, complete digestion of Pro 1 or Pro 2 with leucine aminopeptidase releases no glycine.

Peptide Pro 2 has only one reactive amino acid available for covalent addition of a nucleotide, namely arginine, because the NH2 terminus of Pro 2 is free to react with dansyl chloride. Presumably Pro 1, because of its similarity to Pro 2 in chromatography and electrophoresis, contains the same linkage and is an incomplete digestion product of the same sequence in α. Arginine in fact the amino acid involved in modification, as proven by further digestion of the pronase peptides from 32P-labeled modified α with leucine aminopeptidase. The single labeled peptide observed in this digest, LAP 1 (Table 1), was purified in low yield on paper. It contains only arginine and glycine after acid hydrolysis and reveals NH2-terminal arginine upon dansylation followed by acid hydrolysis. Therefore LAP 1 must be modified arginine. (Normal arginine rather than the modified derivative is found in amino acid analyses and Edman sequences because the modification is lost during acid hydrolysis.)

Chemical Stability of Linkage to Arginine; Possible Structures—The results already presented suggest that modified α contains a covalent bond between some part of adenosine diphosphoribose (or a related ADP-sugar compound) and an arginine in the polypeptide. The bond could reasonably involve either the guanido side chain or the carboxyl group of arginine, and either the phosphates or the terminal sugar residue of the nucleotide. (Release of 5'-AMP from the modified peptides by venom phosphodiesterase (17) rules out a bond directly to the adenosine moiety.)

In order to investigate further where and how the nucleotide is linked, I have examined the chemical stability of 32P in modified α.

For these experiments modified α doubly labeled with 32P and 3H was purified as described under "Experimental Procedures." The purified protein was incubated under the desired conditions and aliquots were withdrawn at intervals, precipitated with cold 5% trichloroacetic acid, collected on Millipore filters, and counted. 32P counts are lost as phosphorus in the nucleotide released from α; 3H counts, in the protein itself, are stable and serve to measure the amount of α precipitated. In all cases a first order rate of loss has been observed for 92 to 95% of input 32P. The remaining 32P is stable to all conditions tested and is probably in fragments of DNA undergoing co-electrophoresis with α on the preparative SDS gel. A plot of hydrolysis kinetics in acid and base at 66° is shown in Fig. 6; the data obtained from these and similar plots is summarized in Table VI.

It is difficult to deduce a specific chemical structure from these data, because neighboring amino acid side chains could alter hydrolysis rates of the protein-nucleotide bond by acid or base catalysis or by steric hindrance. Nevertheless some general conclusions can be drawn from these experiments.

The nucleotide appears to be covalently linked to arginine by a covalent bond between some part of adenosine diphosphoribose and an arginine in the polypeptide.
6.25 plotted on a logarithmic scale in this figure. Incubation in desiccated lyophilized polypeptide was dissolved in 500 μl of either 0.25 N NaOH or 0.25 N NaOH and incubated at 66°C. At the indicated times aliquots were withdrawn to tubes containing 250 μl of 0.1 mg per ml of bovine serum albumin (to act as carrier), precipitated with 2.5 ml of cold 5% trichloroacetic acid, and collected on Millipore HA filters. The filters were washed with 5% trichloroacetic acid and then 95% ethanol, then dried and counted. Data were normalized to a constant number of H counts per min to account for variation in the size of aliquots taken (aliquots taken late in the experiments were larger to allow more accurate 32P counting). 32P data are plotted on a logarithmic scale in this figure. 6, incubation in 0.25 N NaOH. A small amount of 32P was resistant to hydrolysis after 120, 180, and 240 min of incubation. This stable background (6.5% of input 32P) was subtracted from all data before plotting. 7, incubation in 0.25 N HCl. In this experiment, a stable background of 8.0% was subtracted from all 32P data before plotting.

Conclusions can be drawn about the bond being broken. First, the observed stability of the modification to both acid and base is much too great for an anhydride linkage between the phosphate of a nucleotide and the carboxyl group of an amino acid (40). Secondly, a phosphoamide bond to a guanido nitrogen of arginine is unlikely. Phosphorus in the modification is more stable to acid and to pH 4.8 hydroxylamine than to base. This is in marked contrast to the stability expected for a phosphoamide bond, such as the linkage between a lysine ε-amino group and the phosphorus of 5-AMP in the E. coli DNA ligase-adenylate cova lent intermediate (40, 41). Finally, the 32P is far too labile in acid to be linked in a carbon-to-phosphorus bond such as that in 2-aminoethylphosphonic acid (42). Thus the chemical stability of 32P in the α modification seems to rule out a bond directly between phosphorus and arginine. Because linkage through adenosine has already been ruled out, the attachment point for the protein-to-nucleotide bond is very likely within the terminal sugar of the nucleotide. As mentioned, either the guanido or carboxyl group of arginine could be involved. However, the modification (still judging from the 32P stability data) is much more stable to 0.25 N NaOH than expected if a sugar were esterified to the carboxyl group. An ester linkage between the carboxyl group of arginine and a ribose 1-carbon hydroxyl group would be about 3 orders of magnitude less stable under these conditions than is found for the arginine-to-nucleotide bond in α; an ester linkage to a ribose 2- or 3-carbon hydroxyl, about 4 orders of magnitude less stable (43). Among possible alternative structures involving the sugar and the guanido group, one is especially attractive because it is consistent with the simplest hypothetical mechanism for the α modification reaction, i.e. direct transfer of intact adenosine diphosphoribose to α. This structure could be generated from NAD+ and α by a transfer of the adenosine diphosphoribose terminal-ribose 1-carbon from the 1-nitrogen of nicotinamide (in NAD+) to a guanido nitrogen of arginine.

**DISCUSSION**

The Tp-induced α modification reaction, here hypothesized to be a transfer of adenosine diphosphoribose from nicotinamide to a specific polypeptide site, seems to represent a novel utilization of NAD+ in E. coli. The only analogous reaction yet described, ADP-ribosylation of mammalian elongation factor 2 by diphtheria toxin, is also hypothesized to involve a carbon-nitrogen bond, in this case between the adenosine diphosphoribose terminal ribose and the ε-amino nitrogen of lysine (44). Interestingly, this reaction is also catalyzed by a phage-induced enzyme. Diphtheria toxin, excorated by Corynebacterium diphtheriae strains lysogenic for phage β, is known to be the product of a phage gene (45). However, diphtheria toxin and its target are extracellular and apparently not directly involved in the metabolism of the host bacterium. The E. coli RNA polymerase α modification, in contrast, affects a very basic part of the cell's machinery for gene expression, although the physiological effects of the modification on Tp and E. coli transcription are still uncertain. A poly(adenosine diphosphoribose) polymerase present in the nuclei of eukaryotic cells can link the adenosine diphosphoribose moiety of NAD+ to histone proteins in the presence of DNA (46), but this system differs significantly from both the bacterial adenosine diphosphoribosyltransferases. The eukaryotic enzyme exhibits very little specificity in its protein substrates, produces a relatively unstable adenosine diphosphoribose-to-protein bond

**TABLE VI**

| Conditions                  | t1/2 at 37°C | t1/2 at 66°C |
|-----------------------------|--------------|--------------|
| 0.25 N HCl                  | 220          | 18           |
| 0.25 N NaOH                 | 75           | 6            |
| 4 N hydroxylamine, pH 4.8   | 105          |              |
| 4 N sodium acetate, pH 4.8  | >300         |              |

Chemical stability of covalent phosphorus in modified α polypeptide

Data in acid and base were obtained as described in the legend to Fig. 6; half-times for 32P loss were determined from semilogarithmic plots. Data in hydroxylamine and sodium acetate were obtained with α dissolved in 7 M urea (necessary to maintain solubility under these conditions). Modified α-polypeptide doubly labeled with 32P and 3H (prepared as described in Fig. 6) was dissolved in 7 M urea and freed of SDS as described under "Experimental Procedures." The solution of α in urea was lyophilized, then redissolved at 7 M urea and either 4 N hydroxylamine (adjusted to pH 4.8 with HCl) or 4 N sodium acetate, pH 4.8 (as a control). These solutions were incubated and assayed as described in Fig. 6. Again, half-lives for 32P loss were determined from semilogarithmic plots.
induce two sequential changes in CL larger than the normal α-polypeptide, as judged by its decreased tryptic digests of modified CY (48). Moreover, modified CY appears to polymerize additional adenosine diphosphoribose units onto the initial nucleotide residue (reviewed in Ref. 47). Similarly, addition of net charge is chemically similar but not identical with modified CY (Ref. 49). Pronase digests of 32P-labeled “altered” CY purified from cells suggest that T4 may induce a significant decrease in SDS gel mobility much as an apparent molecular weight increase of 1000 per negative charge (19).

A second unanswered question, about the origin and the number of α-modifying enzymes, involves the “alteration” of the α-polypeptides occurring very rapidly after T4 infection even in chloramphenicol-treated cells. As already mentioned, “altered” α is chemically similar but not identical with modified α (Ref. 18 and Fig. 4). "Altered" α appears transiently prior to modified α during normal infection also (18), suggesting that T4 may induce two sequential changes in α. It is possible that two different enzymes are involved, and that the "altering" enzyme (affected by protein synthesis inhibitors) pre-exists in either the phage particle or the host, while the modifying enzyme is synthesized de novo after infection.5 Purification of the components of the in vitro α-modifying system should reveal how many enzyme activities are involved and whether further substitutions of α occur during the modification process.

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6. Extensive screening of T4 mutants has recently revealed two different mutants affecting α: one which “alters” but does not modify α during infection, and another which modifies α but is defective in “alteration” (R. Horvitz, personal communication).
Chemical Structure of a Modification of the *Escherichia coli* Ribonucleic Acid Polymerase α Polypeptides Induced by Bacteriophage T4 Infection
Christopher G. Goff

*J. Biol. Chem.* 1974, 249:6181-6190.

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