Reversible Stalling of Transcription Elongation Complexes by High Pressure

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ABSTRACT We have investigated the effect of high hydrostatic pressure on the stability of RNA polymerase molecules during transcription. RNA polymerase molecules participating in stalled or active ternary transcribing complexes do not dissociate from the template DNA and nascent RNA at pressures up to 180 MPa. A lower limit for the free energy of stabilization of an elongating ternary complex relative to the quaternary structure of the free RNAP molecules is estimated to be 20 kcal/mol. The rate of elongation decreases at high pressure; transcription completely halts at sufficiently high pressure. The overall rate of elongation has an apparent activation volume ($\Delta V^{\ddagger}$) of 55–65 ml · mol^{-1} (at 35°C). The pressure-stalled transcripts are stable and resume elongation at the prepressure rate upon decompression. The efficiency of termination decreases at the ρ-independent terminator tR2 after the transcription reaction has been exposed to high pressure. This suggests that high pressure modifies the ternary complex such that termination is affected in a manner different from that of elongation. The solvent and temperature dependence of the pressure-induced inhibition show evidence for major conformational changes in the core polymerase enzyme during RNA synthesis. It is proposed that the inhibition of the elongation phase of the transcription reaction at elevated pressures is related to a reduction of the partial specific volume of the RNA polymerase molecule; under high pressure, the RNA polymerase molecule does not have the necessary structural flexibility required for the protein to translocate.

INTRODUCTION

It has recently been proposed that the progress of *Escherichia coli* RNA polymerase (RNAP) along a DNA template during transcription involves cycles of sequence-dependent conformational changes of the enzyme within the transcriptional complexes (Zaychikov et al., 1995; Krummel and Chamberlin, 1992; Chamberlin, 1994; Nudler et al., 1994). Cyclic differences in the length of template that is protected by the enzyme are observed in footprinting experiments. These results were interpreted as an inchworm-like movement of RNAP (Krummel and Chamberlin, 1992; Chamberlin, 1994). Based on similar experiments, Heumann and co-workers proposed a similar model (Metzger et al., 1989; Zaychikov et al., 1995). Current evidence suggests that the discontinuous advancement of the polymerase is not only dictated by the dynamics of the enzyme, but that the uneven movement of the enzyme is due to kinetic blocks at particular sequences along the DNA template (Nudler et al., 1994). Between such sites the elongation complex can advance steadily. Recent findings have raised the possibility that pausing and termination are correlated with discontinuous movements of RNAP along the DNA and RNA chains (Nudler et al., 1995; Wang et al., 1995).

Another view of the transcription reaction (the nucleic acid destabilization model) proposes that the thermodynamic stabilities of reaction intermediates control the elongation-termination decision. The free energy of formation of the elongation complex at an average template position is described in this model in terms of three components: an unfavorable free energy for the opening of a DNA bubble from intact duplex DNA, a favorable free energy of binding of the polymerase to the nucleic acid components in the ternary complex (DNA and RNA), and an additional stabilizing free energy due to the formation of the RNA-DNA hybrid within the bubble (Yager and von Hippel, 1991). However, an attractive feature of this model is that it provides a mechanistic and quantitative explanation of the termination process based on a kinetic competition between elongation and termination at every position along the DNA template (von Hippel and Yager, 1991). An analysis of the temperature dependence of termination based on a kinetic competition model suggests that the difference in the activation energies for the elongation and termination pathways is very large and greatly favors elongation, except at termination sites (Wilson and von Hippel, 1994).

Based on the inhibition of enzymatic activity and phosphorescence data, it has been suggested that high pressure may modulate the flexibility of monomeric proteins (Gross et al., 1993; Cioni and Strambini, 1994). Multiple equilibria in which the binding of DNA to protein is coupled to protein-protein interactions have previously been investigated by high pressure on lac repressor (Royer et al., 1990) and nucleosomes (Villas-Boas et al., 1996).

Recently we have introduced pressure as a new variable in the investigation of the stability of RNAP ternary complexes (Erijman and Clegg, 1995); we demonstrated the existence of a broad distribution of RNAP conformers that are differentially sensitive to the level of pressure. These experiments show that RNAP molecules that display essen-

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tially identical catalytic properties in elongation can have large differences in their free energy of subunit association when not actively transcribing (Erijman and Clegg, 1995). In this paper we show that pressure is a useful variable for studying nucleic acid-protein and protein-protein interactions that are coupled to the elongation phase of transcription by RNAP, providing new opportunities for studying the reaction mechanism. We present evidence that the rate of elongation by *E. coli* RNAP is reversibly inhibited by high pressure, and the incorporation of nucleotides into RNA can be halted reversibly on DNA templates at sufficiently high pressures without leading to the dissociation of the RNAP molecule from the template. The solvent and temperature dependencies of the pressure-induced inhibition of the nucleotide incorporation suggest that the dynamics of conformational changes of the enzyme are crucial during elongation.

MATERIALS AND METHODS

Materials

RNAP was purified from *E. coli* by the method of Burgess (1976). The protein was stored in 10 mM Tris-HCl (pH 7.9), 100 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol plus 50% glycerol at −80°C. Plasmid DNA ptR2–4, containing the T7A1 promoter and the tR2 terminator, was a gift from the laboratory of Prof. P. H. von Hippel (University of Oregon). The fragment was amplified by polymerase chain reaction from purified ptR2–4 DNA and two primers defining the ends at positions −441 and +123 (Wilson and von Hippel, 1994). Alternatively, a DNA fragment was prepared by cutting ptR2–4 with *Rsa*I restriction enzyme (Wilson and von Hippel, 1995). T7 DNA was prepared in *E. coli*, and the DNA was separated by standard procedures (Thomas and Abelson, 1966) or purchased from Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany). The T7 DNA was dialyzed into a buffer containing 10 mM Tris (pH 8), 100 mM KCl, and 1 mM EDTA and stored at 4°C. Poly[d(AT)] was purchased from Boehringer Mannheim; the poly[d(AT)] lot had an average length of 5000 nucleotides. Ribonucleotides were purchased from Stratagene (Heidelberg, Germany). Radioactively labeled nucleotides ([α-^{32}P]ATP) were purchased from Amersham (Braunschweig, Germany). The fluorescent analog (γ-AmNS) UTP was synthesized as described (Yarbrough et al., 1979).

Assays of RNAP

Stalled +24 ternary complexes were prepared essentially as described (Wilson and von Hippel, 1994); the sequence of the first 24 nucleotides is given in this reference. Open complexes were formed at the T7A1 promoter by mixing 125 nM RNAP with 50 nM template DNA in transcription buffer (50 mM Tris-HCl at pH 7.9, 100 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, and 1.5% glycerol), followed by incubation for 10 min at 37°C. Transcription was initiated with the addition of 100 μM of the dinucleotide ApU, 50 μM CTP, 50 μM GTP, and 3 μM [α-^{32}P]ATP (750 Ci/mmol), followed by incubation for 3 min at 30°C. The +24 stalled complexes were purified by gel filtration with BioSpin 30 columns (Bio-Rad, Munich, Germany) preequilibrated with transcription buffer.

Transcription reactions at high pressure were performed in sterilized 300-μl polypropylene tubes. A technique that allows mixing of the reaction components at high pressure was used to avoid the atmospheric pressure dead time (Robinson and Sligar, 1994). Radioactive +24 stalled complex (140 μl) was separated from 75 μl of a mixture of four unlabeled nucleoside triphosphates (NTPs) (all at a concentration of 100 μM) by a 70-μl layer of mineral oil. Zirconium oxide beads (0.2 g) (BioSpec Products, Bartlesville, OK) were placed at the bottom of the tube to help break up the oil layer. After the high pressure vessel, which was previously equilibrated at the desired temperature, was assembled, reactions were started by inverting the high pressure tube several times. After the desired time, the pressure was rapidly released and the reaction was stopped by adding an aliquot of the reaction mixture to an equal volume of denaturing loading buffer, incubated at 80°C for 2 min. Low Mg^{2+} concentrations were often used to slow the elongation reaction (Erie et al., 1992). The length distribution of the synthesized RNA transcripts was analyzed by electrophoresis in 15% acrylamide–8 M urea. Dried gels were developed and quantified using a PhosphorImager SF (Molecular Dynamics, Sunnyvale, CA).

The elongation assay using the fluorescent nucleotide analog (γ-AmNS) UTP was carried out as described by Schlageck et al. (1979). The assay solution (400 μl) contained buffer A (10 mM Tris-HCl at pH 7.9, 100 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, and 5% glycerol), 125 nM RNAP, 40 nmol template DNA, and 0.4 mM ATP, and (γ-AmNS) UTP was reduced to 20 μM to extend the linear phase of the reaction. Fluorescence measurements were made with a SLM 8000S fluorometer (Urbana, IL). Excitation was at 360 nm, and fluorescence emission was measured at 500 nm. Under these conditions, cleavage of the α-β-phosphoryl bond of (γ-AmNS) UTP produces an 11-fold increase in fluorescence intensity. The number of nanomoles of (γ-AmNS) UTP consumed during RNA synthesis is therefore

$$\text{nmoles used} = [\text{starting nmoles UTP}] \cdot (F_t - F_o)/(11 \times F_o)$$

where F_0 and F_t are the fluorescence intensities at times 0 and t of the reaction, respectively.

In general, the effects of high pressure on the enzymatic activity are large. The errors given in the figures and tables refer to repetitive experiments.

Viscocity, osmotic pressure, and water molarity values of the glycerol, sucrose, and methanol/buffer mixtures were taken from the literature (Weast and Astle, 1980; Miner and Dalton, 1953).

RESULTS

Stability of ternary elongation complexes

We have examined the effect of high pressure on the stability of *E. coli* RNAP molecules that are participating in elongation ternary complexes stalled by NTP depletion (in this case, no UTP). The DNA template contains the strong T7A1 promoter followed by a sequence of 24 nucleotides free of A (Wilson and von Hippel, 1994). Ternary complexes stalled at position +24 were incubated 30 min at 150 MPa (0.1 MPa = 1 bar) in transcription buffer. After decompression, a mixture with all four NTPs was added. As a control, a binary complex (i.e., in the absence of nucleotides) of RNAP and the DNA template was incubated at the same pressure, and a mixture of four NTPs was added after decompression. Although ~80% of the control RNAP molecules are inactivated at 150 MPa (Fig. 1, *lane 4*; see also Erijman and Clegg, 1995), those RNAP molecules that were actively engaged in elongation (i.e., were stalled at position 24) are not irreversibly inactivated at high pressure (Fig. 1, *lane 3*). The stalled complexes are stable and can resume elongation when the pressure is released. A comparison of the termination statistics (at positions U7 and U8 of the sequence of the tR2 terminator) shows that the exposure of RNAP involved in stalled complexes to high pressure did not affect their termination efficiency when the assay is carried out at atmospheric pressure.

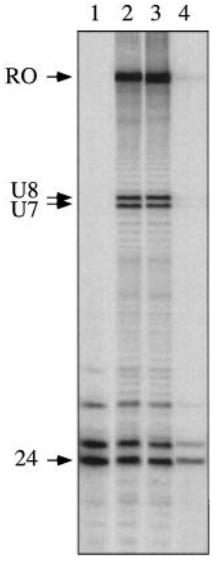

FIGURE 1 Transcription of a polymerase chain reaction-amplified fragment derived from the ptR2–4 template by RNAP. Lanes 3 and 4 have been incubated at 150 MPa. Ternary elongation complexes stalled at position +24 were formed by initiation from the T7A1 promoter (*lane 1*). The complexes were elongated by the addition of the four NTPs before (*lane 2*) and after incubation at 150 MPa (*lane 3*). The RNAP-DNA binary complex was also incubated at 150 MPa for 30 min, after which the transcription reaction was initiated (*lane 4*). The two tR2-terminated positions (U7 and U8) and runoff transcripts (RO) are indicated in the figure.

Pressure-induced decrease of NTP incorporation

A fluorescence assay for monitoring RNA synthesis (Schlageck et al., 1979) was used to follow continuously the in vitro transcriptional activity of RNAP under high pressure. Fluorescence is ideal for continuously following the incorporation of the nucleotides at high pressure in real time. Although no irreversible inactivation of RNAP in a ternary complex occurs at high pressure, the rate of elongation becomes slower, and at a certain pressure the enzyme can be completely halted. Fig. 2 shows that the rate of incorporation of NTPs decreases with increasing pressure. After decompression, the rate of elongation is identical to the rate before the application of high pressure. Even if the compression step is carried out in the presence of heparin (enough to bind all RNAP that is not bound to the ternary complex, 200–500 μg/ml) or rifamycin (enough to inhibit the enzyme completely, ~100 nM), the polymerase is fully active after decompression (data not shown), indicating that RNAP molecules participating in a ternary transcribing complex do not dissociate from the template at high pressure. The σ subunit (which dissociates from the ternary complex after the initiation phase) is not impaired by the elevated pressure; several cycles of transcription are possible when T7 DNA is used as a template and the reaction is allowed to continue at atmospheric pressure after the ternary complex has been subjected to pressures above 150 MPa (Erijman and Clegg, 1996).

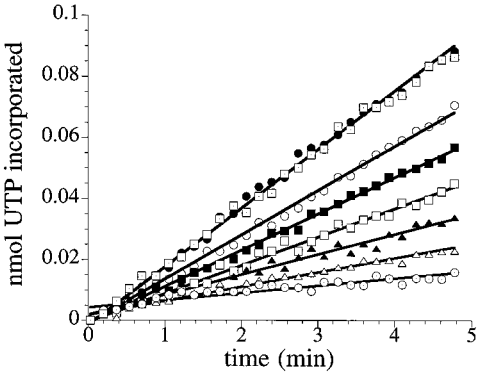

FIGURE 2 Pressure dependence of the catalytic activity of RNAP. Incorporation of AmNS-γ-UTP into nascent RNA was monitored in real time by the increase in fluorescence detected at 500 nm at 0.1 MPa (●), 20 MPa (○), 30 MPa (■), 40 MPa (□), 50 MPa (▲), 60 MPa (△), 80 MPa (⊙), and back to 0.1 MPa (⊡). The template was poly[d(A · T)], and the temperature of the reaction was 35°C.

Pressure-induced decrease in the rate of elongation and in the efficiency of termination

We asked whether the pressure-induced decrease in the rate of incorporation of nucleotides is due to an inhibition of the elongation rate of every RNAP molecule in solution, or whether there is a selective inactivation of only a certain fraction of RNAP molecules that are more sensitive to pressure. To distinguish between these two possibilities, we examined by gel electrophoresis the length distribution of RNA products that were synthesized at elevated pressures, using a DNA template that contains the sequence of tR2 terminator immediately downstream from the strong T7A1 promoter. Fig. 3 shows that the overall rate of elongation is greatly reduced at 100 MPa, for 150 μM and for 1 mM Mg^{2+}. In addition, the synthesis on the template shows preferential retardation at a particular position (indicated with a *star* in Fig. 3; see *lanes 3 and 7*, and compare them to *lanes 2 and 3* of Fig. 1) that is not apparent in the assays carried out at atmospheric pressure (*lanes 2 and 6*). It should be noted that the observability of bands at pause sites is a complex function of the nucleotide incorporation rates and depends on the relative retardation times at different nucleotide sites, as well as on the total time of the assay and the retardation time at any particular site. Obviously, any "pause" site will eventually disappear at sufficiently long times unless it is an irreversible halting site (which would appear as a terminator). However, it appears that the pressure not only slows down the overall rate of elongation, but that the high pressure retards the rate of elongation at the various sites differentially. At lower Mg^{2+} concentration, the polymerase stops within the time of the assay (5′), essentially completely at the position labeled with the * (Fig. 3, *lane 3*). In both Mg^{2+} concentrations the high pressure inhibition is fully released after the pressure is returned to atmospheric pressure (Fig. 3, *lanes 4 and 8*).

Another interesting feature is evident from Fig. 3. When the reaction is carried out under high pressure, there is a

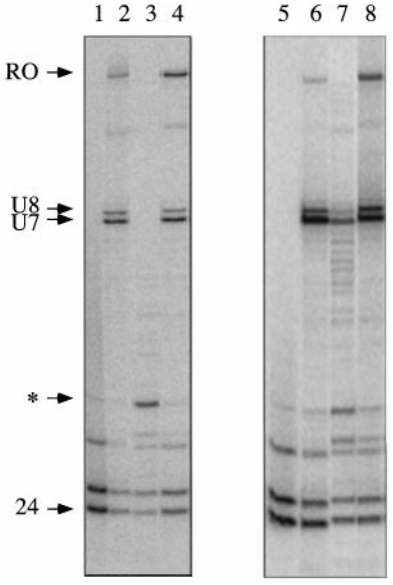

FIGURE 3 Effect of high pressure on the transcription of an *Rsa*I fragment derived from ptR2–4 at 150 μM (*lanes 1–4*) and 1 mM (*lanes 5–8*) Mg^{2+} concentration. Ternary elongation complexes stalled at position +24 (*lanes 1 and 5*) were elongated in the presence of 100 μM of the four NTPs at atmospheric pressure (*lanes 2 and 6*) and 100 MPa (*lanes 3 and 7*). The reactions initially carried out at high pressure (*lanes 3 and 7*) were allowed to continue for an additional 5 min at atmospheric pressure (*lanes 4 and 8*).

consistent decrease in the efficiency of termination (this is clearly seen when the assay is continued after the release of the pressure from the intensity ratio of the RO band to the U7 and U8 bands)). The read-through of the high-pressure-treated samples is increased from 4% to 17% at a Mg^{2+} concentration of 150 μM, and increases from 25% to 35% at a Mg^{2+} concentration of 1 mM. The ratio is difficult to quantify because the value depends on the accuracy of analyzing the gels; but this effect on the read-through of the U7 and U8 terminators is consistently found, and the increase in the read-through fraction can be seen easily by simply comparing the gels directly (e.g., compare *lanes 2 and 6* with *lanes 4 and 8* of Fig. 3). The overall inhibitory effect of high pressure on the catalytic activity of RNAP is similar under standard ionic conditions of 10 mM Mg^{2+} (see below). At low Mg^{2+} concentrations and at atmospheric pressure, an enhancement of the efficiency of termination at the tR2 terminator has previously been observed (Reynolds et al., 1992).

Magnitude of activation volume change

The rate of an elementary chemical reaction step k depends on the pressure according to the expression

$$(\partial \ln k/\partial p)_T = -\Delta V^{\ddagger}/RT \qquad (1)$$

The slope of a plot of $\ln(k)$ as a function of pressure is linearly proportional to the activation volume of the reaction. Despite the complexity of RNA synthesis catalyzed by RNAP, Fig. 4 shows a simple linear dependence of the

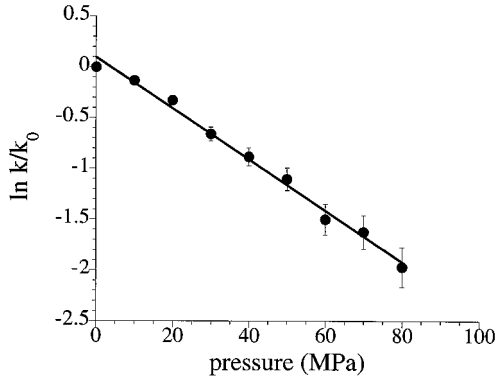

FIGURE 4 Activation volume of the nucleotide addition reaction on poly[d(A · T)] catalyzed by *E. coli* RNAP at 35°C. Each point of the plot represents the slope of kinetic experiments like the one shown in Fig. 2.

logarithm of the rate of RNA synthesis on pressure in congruity with Eq. 1. The overall kinetic rate of the transcription reaction is inactivated by pressure as though there is a rate-limiting reaction step. The linear plot in Fig. 4 also indicates that the apparent $\Delta V^{\ddagger}$ is independent of pressure. The apparent activation volume at 35°C is 55–65 ml · mol^{-1}.

There is no noticeable dependence of $\delta V^{\ddagger}$ on the DNA template

Transcriptional activities on poly[d(A · T)], T7-DNA, and a linearized plasmid containing the lac I promoter are inhibited at elevated pressure; the inhibition of synthesis at high pressure on these templates is also completely reversible after decompression. More importantly, the magnitude of the apparent activation volume change does not depend on the DNA template (Table 1). The synthesis of RNA on the T7-DNA template and on the linearized plasmid DNA template has been shown to proceed eventually to completion after the ternary complex (in a transcribing or stalled phase) has been exposed to high pressure (i.e., until the RNAP molecules reach a terminator on the template) by analyzing the RNA lengths on acrylamide gels (data not shown). Because poly[d(A-T)] does not have a specific promotor or a specific terminator, the same test cannot be made with this polymer—the lengths of RNA are not defined in a unique way; however, the similarity of the $\Delta V^{\ddagger}$ values of all the DNA samples makes it probable that the RNAP has not dissociated from the poly[d(A · T)] template at high pressure. In addition, the RNAP transcriptional activity is fully recovered even after all of the samples have been subjected

TABLE 1 Effect of the template on the HP inhibition of elongation reaction by RNAP at 37°C

Template	$\Delta V^{\ddagger}$ (ml · mol^{-1})
T7 DNA	60.9 ± 3
Poly[d(A · T)]	63.5 ± 2
Plasmid	64.3 ± 3

to very high pressures that are sufficient to completely inactivate the RNAP if the enzyme dissociates from the DNA template (Erijman and Clegg, 1995). This evidence shows conclusively that actively transcribing RNAP molecules are not dissociated from any of the templates (including poly[d(A · T)]) at high pressures.

Dependence of $\Delta V^{\ddagger}$ on salt concentration

Altering the reaction conditions by adding salts is known to produce only a minor effect on the transcriptional activity within the ternary elongating complexes (Neff and Chamberlin, 1980). The efficiency of in vitro termination is essentially unchanged by the concentration of KCl in the range between 150 mM and 1 M (Reynolds et al., 1992; Nudler et al., 1995). The single nucleotide addition apparently involves an isopolar transition state (Erie et al., 1992). If the charge distribution of the activated state is not significantly different from that of the reactants or the products, it would be anticipated that the ionic strength would have relatively little influence on the value of $\Delta V^{\ddagger}$. Table 2 shows that potassium acetate has a negligible effect on the apparent $\Delta V^{\ddagger}$ up to a concentration of 1 M. Protein group transfer hydration between aqueous phases is affected by the ionic composition of the medium. The lack of a salt dependence of $\Delta V^{\ddagger}$ suggests that changes in the exposure of amino acid side chains that carry charges and peptide linkages to water do not contribute significantly to the observed $\Delta V^{\ddagger}$ of the reaction; otherwise, we would expect that because of shielding of polar charged groups on the exposed surface of the protein, the amount of electrostricted water released during catalysis would be reduced (by the presence of the salt), with a concomitant decrease in $\Delta V^{\ddagger}$ (Low and Somero, 1975). Therefore, those solute-solvent interactions in the intermediate complexes of the reaction pathway that are affected by salt are probably not significantly different from those of the reactants for the rate-controlling steps of the reaction.

Dependence of $\Delta V^{\ddagger}$ on the temperature

As shown in Fig. 5, the activation volume increases dramatically as the temperature is raised. The value of $\partial\Delta V^{\ddagger}/\partial T$ is 2.5 ml · mol^{-1} · K^{-1}. The significance of this large effect is discussed below.

Dependence of $\Delta V^{\ddagger}$ on the solvent composition

The importance of the solvent composition in determining the general effect of pressure on reaction rates has been recognized for a long time (Buchanan and Hammann, 1953). Changing the medium by the addition of cosolvents can serve as a probe of the dynamic nature of the processes involved, including solvation and structural changes during catalysis. Mixed aqueous solutions of methanol, glycerol, and sucrose have a modest effect on the nucleotide incorporation activity of a functional ternary complex at atmospheric pressure. Sucrose has the largest effect on the rate of nucleotide incorporation; at the maximum sucrose concentration (53%), the rate is one order of magnitude slower. RNAP activity was measured at increasing pressures for different concentrations of the above-mentioned cosolvents. $\Delta V^{\ddagger}$ was determined from the slope of the linear region of the semilogarithmic plot of rate of elongation as a function of pressure. Fig. 6 shows that only viscosity correlates globally and uniformly with changes in the activation volume ($\Delta\Delta V^{\ddagger}$), but not the osmotic pressure, water concentration, or dielectric constant. As the viscosity increases, the inhibitory effect of pressure upon the rate of the nucleotide addition reaction (i.e., $\Delta V^{\ddagger}$) becomes less significant. At the highest viscosity measured, $\Delta V^{\ddagger}$ is close to zero.

TABLE 2 Effect of the salt concentration on the high pressure inhibition of elongation reaction by RNAP at 30°C

Potassium acetate conc. (M)	$\Delta V^{\ddagger}$ (ml · mol^{-1})
0.1	55.1 ± 2
0.3	54.1 ± 3
1.0	56.7 ± 2

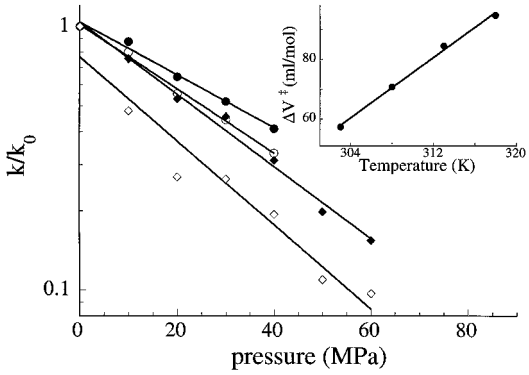

FIGURE 5 Temperature dependence of the activation volume in the synthesis of RNA catalyzed by *E. coli* RNAP. The reaction was carried out at 30°C (●), 35°C (○), 40°C (■), and 45°C (□) and monitored by the increase in fluorescence (at 500 nm) upon incorporation of AmNS-g-UTP into nascent RNA. The template was poly[d(A · T)]. (*Inset*) $\Delta V^{\ddagger}$ versus temperature.

DISCUSSION

RNAP in a ternary complex is exceedingly stable against irreversible pressure effects

We have shown in this work that the ternary complex is remarkably stable at levels of pressure that fully dissociate and irreversibly inactivate RNAP molecules that are free in solution (Erijman and Clegg, 1995). The interactions between the protein subunits, and between the protein and the DNA (and probably importantly, the RNA), are substantially stabilized when RNAP becomes engaged in the elongation phase; the tendency of the actively transcribing enzyme to dissociate is drastically reduced compared to the nontranscribing enzyme. For the nontranscribing enzyme, the level of pressure required to inactivate RNAP is essen-

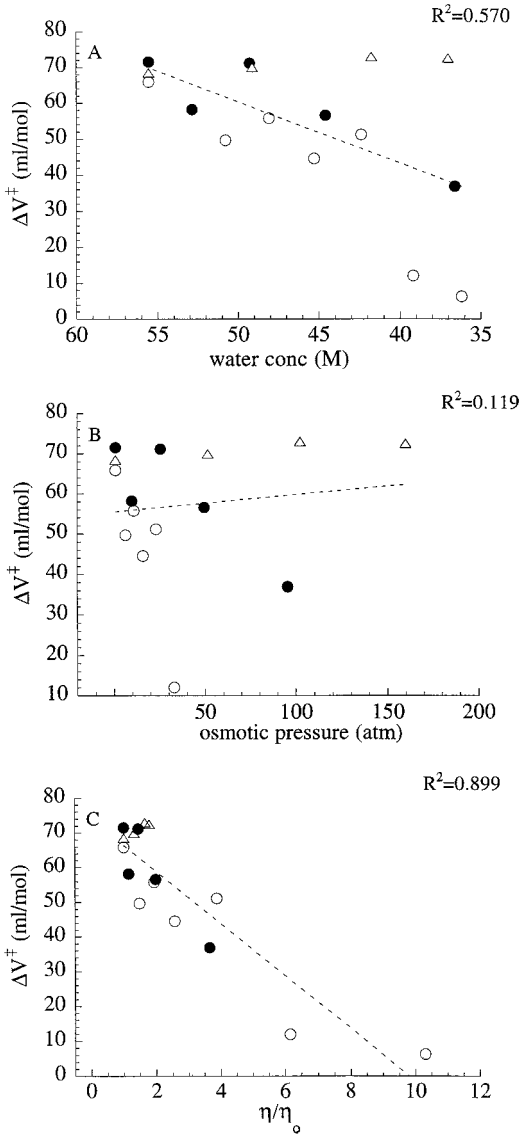

FIGURE 6 Effects of (*A*) water concentration, (*B*) osmotic pressure, and (*C*) viscosity on the $\Delta V^{\ddagger}$ of the elongation reaction. Cosolvents used were glycerol (●), sucrose (○), and methanol (△). Solid lines represent linear fits considering data from all cosolvents as a single data set, with the correlation coefficients indicated in the upper right corner of the figure.

tially independent of whether the RNAP molecule is free in solution, or bound to a DNA molecule (but not in the elongation state) (Erijman and Clegg, 1995). Thus just the imminent presence of the highly negatively charged polyelectrolyte DNA alone does not reduce the propensity of the RNAP subunits to dissociate and to become inactive upon the application of high pressure; the RNAP molecule must be involved in an active ternary complex.

Minimum estimate of the stability of the ternary complex from high pressure studies

It has long been known that a very large stabilization of the RNAP quaternary structure accompanies the formation of the ternary complex (Schäfer et al., 1973; Rhodes and Chamberlin, 1974; Naito and Ishihama, 1975). This process involves large conformational changes (Roe et al., 1985) and the formation of a new set of contacts between the core enzyme and the nucleic acids. A major compositional difference between the ternary complex and the binary RNAP-DNA complexes in the absence of active transcription is the presence of the newly synthesized RNA strand. To evaluate the contribution of the formation of a DNA-RNA hybrid to the free energy of stabilization of the ternary complex, Yager and von Hippel (1991) have calculated the free energy of formation of a 12-bp RNA-DNA hybrid to be approximately -17 kcal · mol^{-1} (37°C in 1 M NaCl) by using the thermodynamic values for stacked nearest-neighbor base pairs from the literature. But there are no reliable experimental values for estimating the free energy contributions to the formation of the ternary complex from conformational changes and from protein-nucleic acid interactions within the transcription bubble; several major assumptions have to be made when estimating these free energy terms (Yager and von Hippel, 1991).

In this section we estimate the extent of stabilization of the RNAP complex structure upon the formation of an elongating ternary complex compared to the binary RNAP-DNA complex by considering the pressure-volume contribution to the overall free energy of the different complexes. We point out that the following discussion assumes that we can apply equilibrium thermodynamic arguments. In addition, one has to keep in mind that the transcription elongation process is a complex reaction system, and we do not know the details of the reaction mechanism. One could choose to compare the two conditions (binary and ternary complexes) in a kinetic sense, in which case we would simply surmise that the dissociation of the ternary complex is so slow (even at high pressure) that the reaction is never observed. This is one possible explanation of our results. Unfortunately, we do not even have values for $\Delta V^{\ddagger}$ for the dissociation reaction of the binary complex. However, by making a simple *Ansatz* that the stabilization is due to an increase in the free energy of stabilization (we have values of $\Delta V^{\ddagger}$, the change in volume of the reaction, for the binary complex), we can estimate the stabilization in the free energy that would be required to hold the complex together. Even though the ternary complex does not dissociate, even at the highest pressure used in this study, we can still set a lower limit to the free energy of stabilization.

The method of estimating the free energy of stabilization is given in the Appendix; it involves knowing the pressure of half-dissociation of each sample, e.g., $P^{\mathrm{T}}_{1/2}$ would be the pressure of half-dissociation of the ternary complex, and $P^{\mathrm{B}}_{1/2}$ that for the binary complex. We do not know $P^{\mathrm{T}}_{1/2}$ because the ternary complex does not dissociate, even at the highest pressure we have tried; however, the minimum value for $P^{\mathrm{T}}_{1/2}$ is the highest pressure we have used (i.e., 200 MPa), and assuming that $\Delta V^{\mathrm{T}} = \Delta V^{\mathrm{B}}$, we can use Eq. A2 to calculate a conservative minimum estimate of the free en-

ergy of the ternary compared to the binary complex to be at least $\Delta\Delta G = 20$ kcal/mol (1 cal = 4.184 J).

This fairly large minimum estimate of $\Delta\Delta G$ is consistent with the requisite to trap the polymerase into a potential well, which is necessary to maintain the highly processive nature of transcription (Yager and von Hippel, 1991); these authors present a model in which they estimate the total free energy of stabilization of the complex to be −19 kcal/mol at 37°C.

Again we emphasize that the above considerations are only strictly valid under the conditions mentioned above. However, if the stability of the ternary complex is controlled mainly by equilibrium considerations (Yager and von Hippel, 1991), then the ternary complex is more stable than the binary complex by more than 20 kcal/mol.

Possible origins of the high stability of the ternary complex

It is interesting to speculate where such a large stabilization of the ternary complex (relative to the RNAP molecule alone or the binary complex) originates. The nascent RNA has often been considered a major factor in stabilizing the ternary complex, and, as mentioned above, the nucleic acid stabilization model (Yager and von Hippel, 1991) attributes a significant portion of the free energy of stabilization to the formation of a DNA-RNA hybrid (approximately -17 kcal $\cdot$ mol^{-1}). On the other hand, it has been shown that RNAP can synthesize RNA progressively, even up to >70°C (Wilson and von Hippel, 1994); however, at this temperature an average 12-bp RNA-DNA hybrid in solution would be substantially destabilized, and for some sequences, a 12-bp duplex would not even be stable. Therefore, it seems that if the RNA-DNA hybrid contributes a dominant stabilization to the ternary complex, the values for ΔH/bp and ΔS/bp corresponding to the formation of the RNA-DNA hybrid are likely to be different from those determined for the formation of a free duplex in solution.

If this DNA-RNA interaction does play a decisive role in the high affinity of the RNAP molecule for the DNA template (thereby protecting the quaternary structure of the RNAP against dissociation at high pressure), then there must also be very strong interactions between the RNAP molecule and the RNA molecule. Such strong interactions of the RNA strand with the protein might also decrease the ΔS/bp corresponding to the melting of the hybrid helix, and this would make the DNA-RNA hybrid effectively stronger.[1] (If the RNA-DNA hybrid melts, and the dissociated RNA strand then interacts with the RNAP molecule, the freedom of the bases would be considerably decreased. This would lead to a decrease in the positive entropy change for the duplex melting reaction. This, according to $\Delta G = \Delta H - T\Delta S$, leads to a less negative ΔG, decreasing the propensity of the duplex to melt.) The inchworm model proposed by Chamberlin (1994) also ascribes a crucial role to protein-RNA interactions for stabilizing the elongation complex. If the RNAP molecule unfolds to some extent during the progress of the polymerase reaction, this may increase the possibility that the newly synthesized RNA strand will interact with the protein, thereby leading to an increased stability. Recently, three sites in RNAP were mapped that are contacted by the RNA in the transcribing elongation complex (Markovtsov et al., 1996). It must also be remembered that the total free energy of stabilization against the dissociation of any of the components does not have to be a linear sum of the separate contributing pairwise interactions. The most weakly bound component, i.e., RNA, DNA, or RNAP, to the ternary complex will control the stability of a functioning ternary complex. Our pressure experiments have shown that the component bound weakest to the complex has a free energy of interaction greater than 20 kcal/mol. This would correspond to a very large equilibrium association constant of $K^{T} \approx 3 \times 10^{14}$ M^{-1}.

The apparent activation volume of the elongation kinetic reaction is large

The changes in volume associated with the formation of an activation complex, $\Delta V^{\ddagger}$, are related to dynamic processes in a system. These dynamics can be related to chemical processes, or to more complex conformational changes, either at the active site or far from it (Low and Somero, 1975). Changes in volume that accompany conformational changes in enzymes during catalysis are thought to arise mainly from two sources: 1) the movement of chemical groups in the protein that modify the density of water in their immediate vicinity into or away from contact with water, and 2) "structural" changes contributed by changes in the volume of the macromolecules themselves (Low and Somero, 1975). We do not know the mechanistic details of transcription or the exact kinetic nature of the measured rate of RNA synthesis. Because we are measuring an average rate, we cannot say from these experiments whether the measured $\Delta V^{\ddagger}$ is sequence independent. There may be positions on the template where the rate of nucleotide incorporation is slower than the average value (e.g., pausing sites); if the degree of pausing is great enough, the pause site can significantly influence the overall rate. One such site is observed directly in the experiment displayed in Fig. 3, and the significance of this sequence-dependent effect is being studied in more detail. Because of the complexity of the transcription reaction, it is unlikely that only one elementary reaction step completely controls the overall pressure sensitivity of the reaction, so k is probably not the rate of an elementary reaction step. However, the influence of different environmental factors on the pressure-induced inhibition of the enzyme allows us to determine some significant characteristics of this process.

Possible sources of the activation volume

Temperature effect

The side-chain flexibilities of a protein are expected to increase as the temperature is increased. We measure a strong dependence of the $\Delta V^{\ddagger}$ on temperature (Fig. 5). If the $\Delta V^{\ddagger}$ that we measure is correlated with a "structural" change during the progress of the reaction, increasing the temperature at which the reaction is carried out might induce a larger $\Delta V^{\ddagger}$ due to a corresponding increase in the expansion of the complex during the nucleotide incorporation at higher temperatures. The conformational flexibility of parts of the protein within the elongating complex may become greater as the temperature is raised. It is conceivable that this may lead to larger activation volume changes during the movement of the enzyme during catalysis. The larger volume of the activated state of the elongation complex relative to the nonactivated state may be related to a decrease in the extent of the tight secondary structure of part of the protein when the enzyme is in the process of incorporating a nucleotide. At this point one should keep in mind that this change in volume (55–60 $ml \cdot mol^{-1}$ at 35°C) corresponds to a very small fraction of the total volume of the complex (~0.2%); however, if the activation volume is operative only in one location of the macromolecular complex, it could represent a significant molar volume change.

Stabilization of the DNA template

The helix-to-coil transition temperature (T_m) of double-stranded DNA is also pressure dependent in a way that makes transcription more difficult at higher pressures. It has been observed experimentally that increasing the pressure raises the T_m of DNA duplexes (i.e., pressure increases the stability of duplex DNA) by ~2°C/kbar for poly[d(AT)] in 150 mM NaCl (Hawley and MacLeod, 1974; Wu and Macgregor, 1993). Because the temperature dependence of the rate of transcription is not large at 30°C, only a minor contribution of increasing pressure on the rate of RNA synthesis would be expected to be due to a stabilization of the DNA template. In addition, the inhibition is more pronounced at higher temperatures, which argues against the proposition that pressure-induced stabilization of double-stranded DNA is a major contributing factor to the decreased rate of elongation.

Chemical reaction or hydration of charges

Another source of pressure inhibition could be associated with the chemical reaction itself. The addition of an NTP (or phosphor transfer) occurs via an SN_2 mechanism (Erie et al., 1992); therefore, these chemical steps are not expected to be affected much by pressure (Asano and Le Noble, 1978). If the inhibitory effect of high pressure were related to the hydration of charges (electrostriction), then we would expect the $\Delta V^{\ddagger}$ to depend on the salt concentration due to the shielding of the charge-charge interactions. However, this is not observed, and therefore it seems that the hydration of charges is also not substantially involved in the observed $\Delta V^{\ddagger}$.

Global structural nature of the $\Delta V^{\ddagger}$

The activation volumes seem to be related to conformational changes in the enzyme without the direct participation of solvents or by the formation and breaking of bonds upon incorporation of NTPs, or both. The strong positive dependence of $\Delta V^{\ddagger}$ on the temperature, which is in contrast to the observed influence of temperature on the pressure effects on most of the ordinary chemical reactions (Asano and Le Noble, 1978), also indicates that the $\Delta V^{\ddagger}$ is related to a global characteristic of the macromolecular structure of the ternary complex.

Correlation of $\Delta V^{\ddagger}$ with the viscosity of the solution: a possibility of more than one pathway of nucleotide incorporation

The apparent correlation of $\Delta\Delta V^{\ddagger}$ with the viscosity of the medium also suggests that the formation and breakage of bonds accompanying the incorporation of NTPs, as well as the concomitant changes in the hydration of participating residues, do not contribute significantly to the observed $\Delta V^{\ddagger}$, so that the measured activation volume reflects mainly global structural changes that increase the volume of the enzyme. The exact nature of the changes involved is currently unknown; however, one possibility is that certain dynamic changes in the structure of the ternary complex (with large $\Delta V^{\ddagger}$, and therefore a pronounced sensitivity to pressure) become less probable at higher viscosities. There may be several possible pathways for the catalytic incorporation of ribonucleotides; as the viscosity is raised, the reaction proceeds more and more by another kinetic pathway that is much slower (i.e., kinetically less effective at atmospheric pressure), but does not proceed with a high activation volume.

The effect of high pressure on the relative rates of elongation and termination

It is unclear why the termination vs. read-through ratio changes after the system has been subjected to high pressure. It is reproducible, but more experiments have to be carried out to determine whether it is a mainly an effect on kinetic constants (i.e., activation volumes) or on stability constants (i.e., reaction volume changes). The fraction of molecules terminating has been discussed in terms of a kinetic competition between the rates of elongation and termination (von Hippel and Yager, 1991). According to the nucleic acid destabilization model (Yager and von Hippel, 1987), a decrease in the elongation rate alone would lead to an increase in termination efficiency, which is not observed in our pressure inactivation studies. By kinetically blocking

structural transitions that presumably involve large amplitude fluctuations, high pressure could decrease the propensity for RNAP to overcome the barrier for signal recognition that makes the termination likely at rho-independent termination sites. If the RNAP molecule is flexible and is capable of partially unfolding its structure to expose molecular groups in the protein structure, an "open-flexible" structure of the enzyme may be important for proper recognition of pausing or termination regulation signals (Nudler et al., 1995; Wang et al., 1995). More work is required to determine whether the major effect of high pressure on the termination efficiency takes place only when the system is exposed to high pressure, or whether the changes in the complex that have taken place at high pressure remain influential upon decompression (at least transiently).

The extraordinary stability of the ternary complex

E. coli RNAP shows an outstanding ability to withstand many perturbing events during the elongation synthesis of RNA chains, and we have shown here that transcribing RNAP is also resistant to exposure to high pressures. Other examples of this extreme stability have been reported. An applied force of 14 pN is necessary to stall polymerase molecules that are actively incorporating nucleosides triphosphate in vitro (Yin et al., 1995). A transcribing RNAP molecule remains associated with the DNA template even when a DNA replication fork passes the RNAP ternary complex, whether the two polymerase processes are running in the same (Liu et al., 1994) or in the opposite (Liu and Alberts, 1995) direction. Heterogeneous mixtures of ternary complexes stalled at different positions of a DNA template are also exceptionally stable at elevated salt concentrations (Beabealashvilly and Savotchkina, 1973; Richardson, 1966; Fukuda and Ishihama, 1974). Well-defined stalled ternary transcription complexes are stable for more than 1 week at salt concentrations as high as 1.0 M K^+ (Arndt and Chamberlin, 1990). These data suggest that the very high stability of the ternary complexes relative to the binary complexes is due mainly to nonionic interactions. It has also been shown that elongation is still possible at very high temperatures, even up to 70°C (Wilson and von Hippel, 1994).

SUMMARY

In conclusion, we suggest that the high-pressure inhibition of the elongation phase of the transcription reaction with RNAP is related to the influence of pressure on the activation volume of the RNAP protein structure that purportedly accompanies the translocation event. The correlation of $\Delta V^{\ddagger}$ with other parameters leads us to the proposition that the kinetic effect of high pressure is related to the flexibility, and therefore the structural fluctuations, of the protein. The flexibility of proteins can be related to their compressibility (i.e., the change in volume in response to variations in pressure), and the compressibility is directly related to the fluctuations in the volume (Cooper, 1976). Pressure-induced reduction in partial specific volume may hinder the necessary structural flexibility that is required by RNAP during active transcription.

APPENDIX: ESTIMATING FREE ENERGY OF STABILIZATION FROM PRESSURE STUDIES

The rationalization of the procedure for estimating the free energy of stabilization from pressure experiments is as follows. The total free energy change representing the overall dissociation of the RNAP ternary complex can be written as

$$\Delta G^{\mathrm{T}} = -RT\ln(K^{\mathrm{T}}) = -RT\ln(K_0^{\mathrm{T}}) + P\Delta V^{\mathrm{T}} \quad \text{(A1)}$$

where the superscript T stands for "ternary," K^{T} is the equilibrium constant of the dissociation reaction of the ternary complex at a particular temperature and pressure, $-RT\ln(K_0^{\mathrm{T}})$ is the contribution to the free energy change minus the pressure-volume term, and $\Delta V^{\ddagger}$ is the molar volume change of the dissociation reaction for the ternary complex. Note that we do not specify here what this dissociation reaction is; it could be the simple (binary) dissociation of one of the RNAP subunits. We just make the assumption that there is some equilibrium reaction that would lead to a dissociation of components that would either cause inactivation or be observable in high-pressure electrophoresis. The same equation can also be written for the binary complex of RNAP/DNA, where the superscript T is then replaced by B. At a pressure $P_{1/2}^{\mathrm{T}}$ corresponding to the half-dissociation point of the ternary reaction, $\Delta G^{\mathrm{T}} = 0$; therefore, $RT\ln(K_0^{\mathrm{T}}) = P_{1/2}^{\mathrm{T}}\Delta V^{\mathrm{T}}$ (see Eq. 2). A similar expression, $RT\ln(K_0^{\mathrm{B}}) = P_{1/2}^{\mathrm{B}}\Delta V^{\mathrm{B}}$, holds for the binary complex. We know $P_{1/2}^{\mathrm{B}}$ and ΔV^{B} from earlier experiments (Erijman and Clegg, 1995). We can use this information to calculate the free energy contribution to the dissociation reaction other than the pressure-volume term for both types of complex. If we assume that $\Delta V^{\mathrm{B}} = \Delta V^{\mathrm{T}} = \Delta V = 200\ \mathrm{ml \cdot mol^{-1}}$ (the numerical value of ΔV^{B} has been determined earlier; Erijman and Clegg, 1995), we can estimate the difference in the free energy of dissociation between the ternary and binary complex at any particular pressure and temperature by

$$\begin{aligned}\Delta\Delta G &= \Delta G^{\mathrm{T}} - \Delta G^{\mathrm{B}} \\ &= RT\ln(K_0^{\mathrm{B}}) - RT\ln(K_0^{\mathrm{T}}) \\ &= (P_{1/2}^{\mathrm{T}} - P_{1/2}^{\mathrm{B}}) \cdot \Delta V.\end{aligned} \quad \text{(A2)}$$

High pressure gives us a way to estimate $\Delta G^{\mathrm{T}} - \Delta G^{\mathrm{B}}$ directly at atmospheric pressure. $\Delta\Delta G \approx RT\ln(K_0^{\mathrm{B}}) - RT\ln(K_0^{\mathrm{T}})$ at atmospheric pressure, because both $P_{1\mathrm{atm}}^{\mathrm{T}}\Delta V^{\mathrm{T}}$ and $P_{1\mathrm{atm}}^{\mathrm{B}}\Delta V^{\mathrm{B}}$ are essentially zero compared to $P_{1/2}^{\mathrm{T}}\Delta V^{\mathrm{T}}$ and $P_{1/2}^{\mathrm{B}}\Delta V^{\mathrm{B}}$; hence the pressure-volume terms do not contribute significantly to the free energy change of the reaction at 1 atm pressure ($P_{1/2}^{\mathrm{T}}$ and $P_{1/2}^{\mathrm{B}}$ are at least several hundred to several thousand atmospheres).

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